The Innate Immune Toll Like Receptor 9 Suppresses Epstein-Barr Virus Lytic Reactivation in Burkitt's Lymphoma Cells: Identification of Important Signaling and Epigenetic Mechanisms

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To my family

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SUMMARY

Burkitt's lymphoma (BL) is the most common cancer in children of equatorial Africa, and is endemic to areas where people are chronically co-infected with Epstein-Barr virus (EBV) and the malaria pathogen *Plasmodium falciparum*. Although the contribution of both pathogens in the oncogenic process remains poorly understood, we showed earlier that the activation of Toll-like receptor (TLR) 9 by hemozoin, a disposal product formed from the digestion of blood by *P. falciparum*, suppresses the lytic reactivation of EBV in BL cells. As latency is a prerequisite for transformation, a clear understanding of the factors that regulate lytic infection is critical. The ability of latent EBV to evade the immune system, and its unique oncogenic potential, might thus be reinforced by co-infection with *P. falciparum*.

In the work presented here, we explore, in EBV-positive BL cell lines as a model system, the TLR9-induced pathway involved in the repression of the viral transcription factor *BZLF1*, the master gene controlling EBV lytic reactivation. Based on previous results, we hypothesized that triggering TLR9-triggering inhibits EBV lytic gene expression through an alternative pathway not yet described.

Moreover, using a luciferase gene reporter system combined with chromatin immune-precipitation assays, we evaluated the effect of TLR9-induction on the histone modifications of *Zp*, the promoter of *BZLF1*, upon lytic reactivation. We observed that, in contrast to the native EBV viral genome, TLR9 triggering did not affect the activation of *Zp* reporter constructs of different length; therefore we hypothesized a distinct chromatin structure of the transfected *Zp* reporter construct compared to EBV's viral genome.

The data presented here provide new insights concerning the signaling pathway linking TLR9 to the *Zp* promoter. Most importantly, our data confirm that histones modifications are the key step in the TLR9-induced suppression of EBV lytic gene expression and that reporter system based assays do not necessarily reflect the physiologic situation.

These findings are of great importance for the development of new therapeutic strategies using the viral lytic reactivation to clear EBV latently infected cancers cells.

ZUSAMMENFASSUNG

Burkitt-Lymphom (BL) ist die häufigste Krebserkrankung bei Kindern in äquatorialen Afrika und ist endemisch in Gebiete, in denen Menschen chronisch mit Epstein-Barr-Virus (EBV) und mit dem Malaria-Erreger *Plasmodium falciparum* infiziert sind. Unseres Labor zeigte, obwohl der Beitrag der beiden Pathogene im onkogenen Prozess nach wie vor schlecht verstanden ist, dass die Aktivierung von Toll-like Rezeptor (TLR) 9 durch Hämozoin, ein Entsorgungsprodukt das aus der Verdauung von Blut durch *P. falciparum* gebildet wird, die lytische Reaktivierung von EBV in BL-Zellen unterdrückt. Da die Latenz eine Voraussetzung für die Transformation von Wirtszellen durch EBV ist, ist ein klares Verständnis der Faktoren, die lytische Infektion regulieren, kritisch. Die Fähigkeit des latenten EBV, dem Immunsystem auszuweichen, und sein einzigartiges onkogenes Potential, könnte somit durch eine Co-Infektion mit *P. falciparum* verstärkt werden.

In der vorliegenden Arbeit untersuchen wir in EBV-positiven BL-Zelllinien als Modellsystem den TLR9-induzierten Weg, der an der Repression des viralen Transkriptionsfaktors *BZLF1,* der Master-Gene der die EBV-lytische Reaktivierung kontrolliert, beteiligt ist. Basierend auf früheren Ergebnissen haben wir die Hypothese aufgestellt, dass die auslösende TLR9-Aktivierung die EBV-lytische Genexpression durch einen alternativen, noch nicht beschriebenen Weg beeinflusst.

Darüber hinaus untersuchten wir die Wirkung der TLR9-Induktion auf die Histonmodifikationen von *Zp*, dem Promotor von *BZLF1*, nach lytischer Reaktivierung mit einem Luciferase-Gen-Reportersystem, kombiniert mit Chromatin-Immunpräzipitationstests. Wir beobachteten, dass im Gegensatz zum nativen EBV-Virusgenom die TLR9-Triggerung die Aktivierung von Zp-Reporterkonstrukten unterschiedlicher Länge nicht beeinflusste. Daher haben wir eine unterschiedliche Chromatinstruktur des transfizierten *Zp*-Reporterkonstruktes gegenüber dem viralen Genom von EBV vermutet.

Die hier präsentierten Daten liefern neue Erkenntnisse über den Signalweg, der TLR9 mit dem *Zp*-Promotor verbindet. Unsere Daten bestätigen, dass Histonmodifikationen der Schlüsselschritt bei der TLR9-induzierten Suppression der EBV-lytischen Genexpression sind und, insbesonders, dass Reportersystem-basierte Assays nicht notwendigerweise die physiologische Situation wiedergeben.

Diese Ergebnisse sind von großer Bedeutung für die Entwicklung neuer therapeutischer Strategien unter Verwendung der viralen lytischen Reaktivierung, um EBV latent infizierte Krebszellen zu klären.

INTRODUCTION

The Epstein-Barr virus

The Epstein-Barr virus (EBV or human herpes virus (HHV)4) is an enveloped double stranded DNA virus belonging to the γL or lymphocryptovirus (LCV) herpesvirus genus (Table 1) (1). EBV is widespread in the human population with more than 90% of the adults positive for serum IgG antibodies against the viral capsid antigen (VCA) complex of EBV (2). The virus is usually transmitted *via* saliva and *in vivo* mainly targets B lymphocytes and epithelial cells (3). Primary infection usually occurs during childhood and is often asymptomatic in toddlers. However, with the improvement of the hygienic conditions the primary infection may be delayed until adolescence or adulthood, resulting, in up to 75% of the cases, in the most frequent clinical manifestation of EBV infection called infectious mononucleosis (IM) or 'kissing disease' (4, 5). The IM symptoms, similar to the symptoms caused by flue or HIV infection, include fever, fatigue, enlarged spleen, tonsils and lymph nodes, sore throat, muscle weakness and abdominal pain and are due to a vigorous immune hyperactivation (5). Upon primary infection, the host remains a lifelong carrier of EBV.

The 184 kb double stranded DNA genome of EBV is packaged in a capsid as a linear molecule surrounded by a lipid bilayer in the viral particles. Upon infection of a host cell the viral genome undergoes a circularization in the terminal repeat regions coupled to a chromatinization step. Once the viral latency is established in the host cell, the virus can replicate as an episome during the cellular mitosis using the host cell's replication machinery. However, periodically, upon certain stimuli, the viral productive lytic state is re-activated leading to the horizontal transmission of the virus from cell to cell and from host to host. During lytic replication, the expression of two immediate early proteins called Zta (also known as ZEBRA, EB1, or Z) and Rta (also known as R) initiates the lytic transcription cascade of different classes of viral lytic genes (early and late) resulting in the production of infectious EBV particles and the death of the cell (6).

Table 1: Human herpesvirus taxonomy, nomenclature and main cellular tropism (adapted from (1)**)**

Designation	Trivial name (and acronym)	Herpesvirus subfamily; genus	Primary target cells
HHV-1	Herpes simplex virus-1 (HSV-1)	Alpha; Simplexvirus	Mucoepithelia
HHV-2	Herpes simplex virus-2 (HSV-2)	Alpha; Simplexvirus	Mucoepithelia
HHV-3	Varizella-zoster virus (VZV)	Alpha; Varicellovirus	Mucoepithelia
HHV-4	Epstein-Barr virus (EBV)	Gamma; Lymphocryptovirus	B lymphocytes and epithelial cells
HHV-5	Human cytomegalovirus (HCMV)	Beta; Cytomegalovirus	Epithelia, monocytes and lymphocytes
HHV-6A/-6B	Roseola virus (HHV-6A/-6B)	Beta; Roseolovirus	T lymphocytes
HHV-7	Roseola virus (HHV-7)	Beta; Roseolovirus	T lymphocytes
HHV-8	Kaposi's sarcoma-associated herpesvirus (KSHV)	Gamma; Rhadinovirus	Probably lymphocytes and ephitelia

EBV associated malignancies

In 1958, Denis Burkitt, a British surgeon, reported an aggressive cancer caused by the uncontrolled multiplication of white blood cells. This cancer, common in young children across central Africa, was later named after him as Burkitt's Lymphoma (7). Burkitt noticed a strong link to environmental conditions; the children living in rainy areas with a high temperature year-round were more affected by the cancer than others. This geographical pattern, very similar to the pattern of malaria, led him to believe that an insect-borne virus transmitted the lymphoma. This theory greatly excited a young cancer research doctor called Anthony Epstein. He had been working on the Rous sarcoma virus causing tumors in chickens and was determined to be the first to find a cancer-causing virus in humans. In 1964, Anthony Epstein with the help of Yvonne Barr and Burt Achong could, for the first time, show viral particles in lymphoblasts from Burkitt's Lymphoma (8). This virus, later called EBV, was the first human virus to show the capacity to induce lymphoproliferation and cancers (9).

As already mentioned, EBV is a ubiquitous infectious agent infecting more than 90% of the world's population. A vast majority of the infected individuals do not show any significant symptoms during their life. However, since EBV's discovery it has been shown that EBV is clearly an important factor in the development of multiple human cancers. Moreover, association with neoplasia is a feature shared with other γ-herpesviruses like Kaposi's sarcoma-associated-herpesvirus (KSHV), for example (10). Indeed, next to B cells lymphoproliferative diseases and lymphomas, including Burkitt's lymphoma, Hodgkin's lymphoma and post-transplant lymphoproliferative diseases (PTLD), EBV also causes tumors from other cell types such as T-cell lymphomas (angio-immunoblastic T-cell lymphoma, extranodal nasal-type natural killer /T-cell lymphoma), nasopharyngeal cancer (NPC) and a subset of gastric cancers (11). Therefore, one can raise the question of what are the unique aspects of infection in the people who develop cancers. The study of EBV and its associated tumors points out that many of the malignancies develop: 1) in immunosuppressive patients who are deficient in T-cell mediated immunity, following bone marrow or solid organ transplantation, or upon HIV infection, or 2) have an endemic pattern of incidence (12). Given the growth transforming properties of EBV *in vitro* (13), the association of the virus with post-transplant lymphoma or AIDS-associated lymphomas, in situations were the immune control of EBV-infected host cells is lowered, is not surprising. Next to that, Burkitt's lymphoma is endemic to subequatorial Africa, but also Brazil, Egypt and Papua New Guinea; NPC occurs with high incidence in southern China and with elevated incidence in Inuit populations and in Mediterranean Africa; EBV-associated T-cell lymphomas develop with increased frequency in Taiwan and Japan and EBV-associated parotid gland tumors occur most frequently among Inuits. These observations suggest specific interactions between environmental, genetic, and viral factors (14). All these tumors are characterized by the presence of multiple extrachromosomal copies of the circular EBV genome in the tumor cells and expression of EBV-encoded latent genes, which appear to contribute to the malignant phenotype (15). Different types of tumors are characterized by different patterns of EBV gene expression, the more immune competent the host, the less genes EBV expresses to evade immune recognition (Table 2) (16).

Table 2: EBV associated malignancies and characteristic latent gene expression patterns (adapted from (16)).

Malignancy	EBV-association (%)	Latent EBV gene expressed	Type of latency
Burkitt's lymphoma			
Endemic	100		
Sporadic	15-85	EBERs, EBNA1	I
AIDS-associated	30-40		
Hodgkin's lymphoma			
Mixed cell; lymphocyte depleted	60-80	EBERs, EBNA1, LMP1,	II
Nodular sclerosing	20-40	LMP2a-b	
Post-transplant lymphoproliferative disease			
Immunodefficient	100	EBERs, EBNA1-6, LMP1, LMP2a-b	III
Post-transplant	>90		
AIDS-associated	>80		
Nasopharyngeal carcinoma			
Undifferentiated, non-keratinizing	100	EBERs, EBNA1, (LMP1 [a]), LMP2a-b[a]	I/II[a]
Undifferentiated, keratinizing	30-100		
Gastric carcinoma			
UCNT	100	EBERs, EBNA1, (LMP2)[b]	I[b];(II)
Adenocarcinoma	5-15		
NT/T-cell lymphoma			
VAHS-associated	100	EBERs, EBNA1, (LMP1)[c],	II[c];(I)
Nasal	100	LMP2	
Leiomyosarcoma			
Immunodefficient	100	(EBERs, EBNA1,	(Type III
Post-transplant	100	EBNA2, LMP1, LMP2a-b)	suggested)
AIDS-associated	100	(17)	(17)

AIDS = acquired immunodeficiency syndrome; UCNT = undifferentiated carcinomas of the nasopharyngeal type; VAHS = virus-associated hemophagocytic syndrome; EBERs = EBV-encoded RNAs; EBNA = Epstein-Barr nuclear antigen; LMP = latent membrane protein; [a] = intermediate latency type with variable levels of LMP expression (18); [b] = latency type I and 40% of the cases show LMP2 expression (19); [c] = latency pattern follows type II with LMP1 expression restricted to few cells (20).

Burkitt's Lymphoma

Our research group focused on one of these EBV associated malignancies: the Burkitt's lymphoma.

Burkitt's lymphoma (BL) can be classified into three forms: endemic (eBL), sporadic (sBL) and HIV-associated BL (21). The eBL harbors EBV in over 95% of tumors. Distribution of eBL in sub-Saharan and equatorial Africa coincides with the distribution of endemic *Plasmodium falciparum* malaria suggesting *P. falciparum* to be responsible for the tumor (7, 22). There, BL has an incidence of 5–10/100 000 children and accounts for up to 74% of childhood malignancies. BL often manifests as tumors of the jaw or kidneys, it may also occur in the abdomen, ovaries, facial bones and other extranodal sites. In contrast to eBL,

sBL is rarely associated with EBV, has a low incidence rate worldwide (1-2% of adult lymphoma in Western Europe and America) and most frequently involves tumors of the abdomen. A third BL tumor form, where only about 30% of the tumors are EBV positive, develops in HIV carriers subject to severe immunosuppression coincident with the onset of AIDs. Finally BLs that display intermediate associations with EBV have also been documented in Egypt and Brazil, where up to 87% of the tumors are EBV positive (21, 23).

All BL tumors, including the minority that are not EBV-associated, are characterized by a chromosomal translocation involving the immunoglobulin gene loci on chromosomes 14, 22 or 2 and the *c-myc* oncogene on chromosome 8 resulting in its constitutive activation (24–26) that induces a rapid proliferation and tumor formation. Moreover, there is also evidence for genetic alterations of the tumor suppressor *p53* gene in 60% of BL cases (27), and of the putative tumor suppressor gene retinoblastoma-like 2 (RB2) in most cases of eBL and sBL (28, 29). These mutations indicate a certain genetic instability caused or favored by the presence of *P. falciparum* and EBV although in the case of sBL other factors are likely to play a role.

The eBL tumor derives from cells that have undergone a somatic hypermutation, a feature of the germinal center (GC) reaction during B-cell activation and differentiation. The breakpoint in the Ig gene, to which *c-myc* is translocated in eBL, occurs at the V(D)J region, suggesting that translocation occurs during V(D)J recombination. The J segments flanking *c-myc* translocated breakpoints typically exhibit deletions and/or additions of base pairs characteristic of normal Ig V(D)J segment rearrangement. This is a process catalyzed by B-cell specific V(D)J recombinase activating enzymes RAG-1/2 which are expressed in both pre-B cells and GC B cells (30). Next to RAG-1/2, the *c-myc* translocation might also be mediated through the activation-induced cytidine deaminase (AID), which is highly expressed in the GC as it is responsible for the somatic hypermutation and class switch recombination of immunoglobulin genes as they undergo affinity maturation (31–33). In contrast to eBL, the chromosomal breakpoint in sBL and HIV-associated BL occurs most commonly in the class switch region. Somatic hypermutation and class switching are events that are normally confined to GC B cells. GC centroblast markers are expressed on BL cells and BL-associated factors such as holoendemic malaria, chronic HIV infection and EBV infection, stimulate the proliferation of the B-cells in germinal center. All these observations indicate that BL progenitor cells arise from B cells subjected to chromosomal rearrangements in the GC (15,

34). Thus, the GC events are responsible for the generation of B-cells carrying genetic alterations.

Although the exact underlying mechanism linking EBV infection of B cells to the emergence of malignancy remains poorly understood, there are several potential explanations: - EBV could promote the genomic instability, deregulate telomere functions, and induce DNA damage in infected cells. That could increase the risk of genetic accidents as for example *the c-myc* translocation (35), - EBV could, through either the EBNA1 protein, through EBER transcripts, through the suppression of pro-apoptotic Bim function or through epigenetic modifications, provide a resistance to the apoptosis induced by the *c-myc* translocation, (36–39). Thus, EBV would favor tumor cell survival. Finally, - EBV could drive the tumorigenesis, through its encoded microRNAs that have been identified in BL. Indeed, BHRF1-3 is suspected to regulate the immune-surveillance of the host transformed cells (40), BART-6-3p acts, in synergy with the cellular miR143, as a pro-oncogene by inhibiting the expression of the phosphatase and tensin homolog (PTEN) tumor suppressor (41) and different BARTs decrease CASP3 apoptotic activity (42).

The contribution of *P. falciparum* to the development of eBL is not well understood, but several studies suggest multiple immunomodulatory effects and B-cell activation (43–45). Malaria parasite DNA conjugated to hemozoin, an insoluble crystalline form of heme formed from the digestion of hemoglobin, is a ligand that activates the Toll-like receptor 9 (TLR9) signaling. The activation of TLR9 in turn increases the transformation of B-cell by EBV *in vitro* (46, 47). Therefore, chronic stimulation of TLR9 by *P. falciparum* DNA might increase EBV-induced B-cell transformation *in vivo*. Likewise, TLR9 activation by its synthetic ligand oligodeoxynucleotide (ODN) CpG, or hemozoin, inhibits EBV lytic reactivation *in vitro* in eBL cells (48). These concepts will be presented more in details later in the chapter dedicated to TLR9. Furthermore, a malaria membrane protein increases the EBV genome copies in eBL and peripheral blood mononuclear cells (PBMCs) by an unknown mechanism (49). In the end, more than 50 years after first description of the virus, despite the huge progresses that have been made, we still do not exactly know by which mechanisms the different factors influence the development of BL.

EBV Life Cycle

EBV is transmitted via saliva and infects new hosts in the oropharynx. Once passed the epithelial barrier, it gains access to B-cells within the lymphoid organs. *In vivo*, depending on the location and differentiation state of the infected B cell, EBV expresses three different gene expression programs associated with latent infection, which are: - the growth program, also called latency III, in which all nine known latent proteins are expressed; - the default program also called latency II, in which a restricted set of three latent proteins are expressed; - and the latency program, also called latency I, in which only few if any latent genes are expressed (Table 3).

The very limited number of viral genes expressed during the latency I program enables the virus to avoid immune recognition and to persist *in vivo* in a quiescent state within the memory B cells that circulate in the peripheral blood (50–56). During a persistent infection between 0.5 and 50 per 10^6 memory B cells in the blood are latently infected with EBV (57).

Table 3: Latency transcription programs (adapted from (58)**)**

Latency gene expression program	Genes expressed	Proposed function
Latency program / latency I	EBNA1 (LMP2A*) EBER1-2 BARTs	Allows persistence of the virus in resting recirculating memory cells in a way that is non-pathogenic and not detectable by the immune system
Default program / latency II	EBNA1 LMP1 LMP2A EBER1-2 BARTs	Provides necessary survival signals for: (i) infected lymphoblasts to differentiate into memory, and (ii) maintenance of persistently infected memory cells
Growth program / latency III	EBNA1-6 LMP1 LMP2A-B EBER1-2 BARTs	Activates a resting B cell to become a proliferating lymphoblast

BARTs = BamHI A rightward transcript; EBERs = EBV-encoded RNAs; EBNA = Epstein-Barr nuclear antigen; LMP = latent membrane protein; * = The transcript for LMP2A is often detected in these cells.

The *in vitro* transformation of B cells depends on the viral growth program and on the expression of the nine viral latent proteins that are under the control of the transcription factor EBV nuclear antigen 2 (EBNA2) (59).

In vivo, in healthy individuals, the virus is continuously controlled by the immune system. Stable levels of cytotoxic T lymphocytes (CTL) and serum antibodies to lytic and latent proteins control and drive the three latent gene expression programs (60, 61). However, due to the ability of EBV to transform resting B-lymphocytes into activated proliferating lymphoblast *in vitro*, it is not surprising to observe that in combination with immunosuppressive conditions or in combination with environmental and genetic cofactors, EBV is associated with a number of neoplastic diseases, including lymphomas and carcinomas (16, 62).

Finally, latent EBV can reactivate and switch to its lytic form. Upon lytic reactivation the immediate-early proteins Zta (also known as ZEBRA, EB1, or Z) coded by the *BZLF1* gene and Rta (also known as R) coded by the *BRLF1* gene are the first viral products expressed. Zta and Rta are viral transcription factors that play multiple roles in the lytic replication of EBV; they activate in particular the lytic gene expression cascade. The expression of immediate-early genes *BZLF1* and *BRLF1* is followed by expression of early and late gene that leads to the formation of new viral particles and to the lysis of the host cell (6, 63). *In vivo*, the lytic reactivation of EBV occurs when a memory B cell is stimulated by its specific antigen and differentiates into a plasma cell (6, 64). On the other hand, *in vitro*,

several chemical and biological agents induce EBV lytic reactivation. These include the treatment of the latently infected cells with anti-immunoglobulin (anti-Ig), with transforming growth factor beta 1 (TGFβ1), with protein kinase C (PKC) agonists 12-O-tetradecanoylphorbol-13-acetate (TPA), with DNA methyltransferase inhibitors (e.g., azacytidine), with histone deacetylase (HDAC) inhibitors like sodium butyrate (SB) or trichostatin A (TSA), with addition of Ca2+ ionophores, and with infection with human herpesvirus 6 (65, 66). The differences in the stimulation time and in the response time required to observe *BZLF1* and *BRLF1* expressions, indicates that these agents operate through different mechanisms and different signaling pathways. Of these stimulations, the most relevant physiologically is the cross-linking of the B-cell receptor (BCR) with anti-Ig that mimics the mechanism of the antigen stimulation of a latently infected memory B cell, the germinal center reactions and/or the differentiation into a plasma cell (67, 68).

During the lytic phase, all the viral genes are expressed in a sequential manner that leads to the production of new infectious viral particles, to the death of the host cell, to the infection of new susceptible cells and to the spread to new individuals (6, 58, 69). This event closes the viral life cycle and underscores how well EBV evolved and adapted to make use of the biology of normal B cells.

The B-cell receptor pathway

Cross-linking anti-immunoglobuling (anti-IgG) antibodies induce the lytic expression and reactivation of many EBV positive BL cell lines (63) and therefore, the BCR is a key element in analyzing the switch between latent and lytic EBV infection.

The surface immunoglobulins (Ig) that form the BCR are coupled by a non-covalent bond between the Igα and the Igβ proteins. Each of the subunits contains a single immunoreceptor tyrosine-base activation motif (ITAM). Upon the cross-linking of an antigen, the BCR aggregates and undergoes conformational changes (70) that lead to LYN-mediated phosphorylation of the ITAMs. The phosphorylation of ITAMs creates a docking site for SYK and the recruitment of more LYN kinases (71). Four major signaling pathways are activated, which include the phospholipase C (PLC), the Rho family of GTPases, the rat sarcoma protein RAS and the phosphatidylinositol-3-kinase (PI3K) (72, 73). They recruit MAP-kinases like the extracellular signal-regulated kinase (ERK), the c-jun NH2-terminus kinase (JNK/SAPK) or p38 MAPK that phosphorylate different transcription factors including

Elk1 and *c-Myc* for ERK, c-Jun and ATF2 (activating transcription factor 2) for JNK and ATF2 and MAX for p38 MAPK, which lead to the transcription of a variety of genes.

The BCR signal transduction is a complex network of regulatory signaling cascades operating with other stimuli to control the functions of B cells, the above-described process is a very simplified general model which does not take into account the modularity of this process based on the context of lymphatic tissue or peripheral blood and on maturation stage of the B cell (74).

Interestingly, EBV encodes a latent membrane protein, LMP2A, which mimics BCR and disrupts its association with downstream kinases (75). Latently infected cells that express high levels of LMP2A are resistant to lytic reactivation via BCR engagement.

EBV's immediate-early lytic promoter *Zp* regulatory elements

Zta is a sequence-specific DNA-binding protein, member of the bZIP family of leucine-zipper transactivators. It binds to methylated Zta-responsive-elements (ZRE) dependent on the presence of methylated CpGs (76). During primary infection Zta is transiently expressed but no lytic reactivation occurs, in contrast, lytic reactivation occurs from latent viral genome, which is heavily methylated. Therefore, methylation of viral DNA seems to be a key determinant of lytic activation (77, 78). By binding to the viral DNA origin of lytic replication, ori-lyt, and down regulating the latency-associated promoters Cp and Wp, Zta directly contributes to the EBV replication. Moreover Zta serves as a transcriptional transactivator of its own methylated promoter *Zp* and of other EBV lytic promoters including Rp, the promoter of Rta, and cellular promoters (6, 63). Its expression alone is sufficient to trigger the entire lytic cascade (79, 80), which indicates Zta to be the major immediate-early protein in EBV.

As already mentioned, upon BCR engagement one or more of the phosphotyrosine kinases, BTK, SYK and LYN are activated, followed by the phosphatidylinositol-3-kinase (PI3K), PKC and mitogen-activated protein kinases (MAPK), which transduce the induction signal to *Zp* (81–84).

The regulation of *Zp* has been investigated extensively. Several cis-DNA regulatory elements have been described: they are numbered ZI to ZV, in addition to SMAD-binding elements (SBEs) and the HIF response elements (Table 4). Most of the positive regulatory

elements promoting *Zp* expression seem to be located within the nucleotide (nt) region -221 to +12 relative to the promoter's transcription initiation site. Indeed the -221 *Zp* region coupled to a luciferase gene was sufficient to activate the luminescent signal upon BCR cross-linking in gene reporter assays (85).

In latently infected cells, in the absence of lytic inducing stimuli, suppressor factors such as MEF-2D, ZEB1 or ZEB2 play a role in compacting the local chromatin structure to prevent binding of constitutively expressed Zta and inhibit the activation function of Zta (86–88). Three cis elements that negatively regulate *Zp* have been described within the nt -221 to +12 region relative to the promoter's transcription initiation site, they are called ZV, ZIIR and HIε, and 5 cis elements have been described within the nt -551 to -222 region, they are called ZIV and HIα-HIδ (89) (Table 4). The protein ZEB1 and ZEB2 which are zinc finger E-box-binding proteins bind to the ZV region (90, 91). MEF-2D binds to the ZI region and can associate with HDACs or with histone acetyltransferases (HATs) depending on its the phosphorylation state.

Table 4: *Zp* promoter regions, their locations and their functions (adapted from (92))

Name	Motif	Location	Function
ZI (A-D)	AT-rich	Within -221 to +12 region	Can bind the transcription factors Sp1, Sp3 and MEF2D
ZII	CRE-like motif	Within -221 to +12 region	Can bind CREB, ATF family members, C7EBPs, and the AP-1 family of transcription factors
ZIII		Within -221 to +12 region	Contains multiple binding sites for Zta itself
ZIV		Within -551 to -222 region	Transcriptional silencing element
ZV		Within -221 to +12 region	Transcriptional silencing element, binds ZEB1
ZIIIR		Within -221 to +12 region	Transcriptional silencing element
HIε		Within -221 to +12 region	Transcriptional silencing element
HIα-HIδ		Within -551 to -222 region	Transcriptional silencing element

As we have seen many *Zp* regulatory elements have already been described but still not all have been identified, especially the lytic suppressor elements. The role of the

epigenetic mechanisms to regulate EBV's latency and lytic reactivation will be discussed in another part of this introduction

Toll-like receptor 9 and its signaling in B cells

TLRs and their role in the innate immunity

The innate immune system acts as a first line of defense by sensing invading pathogens. It mediates direct antimicrobial effects and delivers important immunoregulatory signals to activate the defense mechanisms mediated through preexisting immune cells. The toll-like receptors (TLRs) are an essential element of the innate immune system. TLRs are members of the pattern-recognition receptors (PRRs) that also comprise the membrane-bound C-type lectin receptors (CLR) detecting fungi (93), the cytoplasmic nucleotide-binding oligomerization domain receptors (NRL) detecting bacterial products (94) and RIG-like receptors (RLR) detecting viral nucleic acid (95). The role of TLRs, as for the other PRRs, is to recognize structurally conserved molecules; the so-called pathogen associated molecular patterns (PAMPs) that include lipids, lipoproteins or nucleic acids, expressed by viral, bacterial, parasitic or fungal pathogens (96) and the so-called danger-associated molecular patterns (DAMPs), that are endogenous molecules released by necrotic or dying cells (97–99). Upon recognition of their specific ligands (Table 5) a signaling cascade is activated, leading to the expression of interferons (IFNs), pro-inflammatory cytokines and effector cytokines, which have anti-microbial effects. Moreover, by activating somatic hypermutation or by modulating the class-switch recombination in B cells, TLRs activation plays an important role to direct and shape the slower adaptive immune response upon pathogen invasion (100, 101).

TLRs are type I transmembrane proteins characterized by an N-terminal leucin-rich repeats (LRRs) extracellular domain responsible for the ligand recognition, a single transmembrane domain and a C-terminal toll-interleukin 1 receptor (TIR) cytoplasmic domain that binds distinct adaptor molecules important for the signal transduction (102).

In humans, 10 different TLRs have been identified and ligands for all TLRs except TLR10 have been established (Table 5). Moreover, the repertoire of TLRs activating ligands is extended by the ability of TLRs to heterodimerize with one another; for example TLR2 and TLR6 heterodimers are required to recognize diacylated lipoproteins, or TLR2 and TLR1 dimers are required to recognize triacylated lipoproteins (103).

Table 5: Toll-like receptor location, ligands, signal adaptors and production (adapted from (96, 104)**)**
Mo: monocytes, Mϕ: marcophages, DC: dendritic cells, MC: Mast cells, B: B cells, T: T cells, IE: Intestinal epithelium, HSP: heat shock protein, HMGB: high mobility group protein, IC: Inflammatory cytokines

Receptor	Immune Cell Expression	PAMPs	DAMPs	Signal Adaptor	Production
TLR1-TLR2	Cell surface Mo, Mϕ, DC, B	Triacylated lipoproteins, Peptidoglycans, Lipopolysaccharides	HSP 60, 70, Gp96, HMGB1, Proteoglycans	TIRAP, MyD88/Mal	IC
TLR2-TLR6	Cell surface Mo, Mϕ, MC, B	Diacylated lipoproteins	HSP 60, 70, Gp96, HMGB1, Proteoglycans	TIRAP, MyD88/Mal	IC
TLR3	Endosomes B, T, NK, DC	dsRNA, tRNA, siRNA	mRNA, tRNA	TRIF	IC, type 1 IFN
TLR4	Cell surface/endosomes Mo, Mϕ, DC, MC, IE	Lipopolysaccharides	HSP22, 60, 70, 72, Gp96, HMGB1, Proteoglycans, Fibronectin, Tenascin-C	TRAM, TRIF, TIRAP, MyD88/Mal	IC, type 1 IFN
TLR5	Cell surface Mo, Mϕ, DC, IE	Flagellin		MyD88	IC
TLR7	Endosomes Mo, Mϕ, DC, B	ssRNA	ssRNA	MyD88	IC, type 1 IFN
TLR8	Endomes Mo, Mϕ, DC, MC	ssRNA	ssRNA	MyD88	IC, type 1 IFN
TLR9	Endosomes Mo, Mϕ, DC, B, T	CpG DNA	Chromatin IgG complex	MyD88	IC, type 1 IFN
TLR10	Cell surface Mo, Mϕ, DC	Profiling-like proteins		MyD88	IC

TLRs can be divided into two groups based on their cellular location. TLR1, 2, 4, 5, 6, and 10 are located on the surface of the plasma membrane. Thus they are perfectly designed to recognize hydrophobic structures of the invading pathogens, such as lipoproteins, lipopeptides and lipopolysaccharides. TLR3, 7, 8 and 9, on the other hand, are located in endosomes and liposomes within the cells (Table 5). They are well suited to recognize the less easily accessible ligands like for example RNA and DNA, which are the most potent immune stimulatory components of viruses (104).

TLR9 recognizes unmethylated CpG motifs that are relatively rare in vertebrate DNA but abundantly present in herpesviral genomes and senses members from all herpesvirus subgroups (105, 106) including EBV (107). The ligand of TLR3 is double-stranded RNA, a molecular pattern that is not inherently present in herpesvirus genomes but can be generated as intermediate structures during replication and act as TLR3 agonist (108). In the case of EBV, latency-associated EBV-encoded small RNAs (EBERs) are an additional ligand for TLR3 (109). Finally, TLR7 is responsive to single-stranded RNA and has been reported to complement TLR9 signaling to generate an efficient immune response against murine Cytomegalovirus even though the exact nature of the viral agonist is not known (110).

TLR9 expression and signaling in B-cells

The human B-cells, the cells we focused on in this study, commonly express TLR1, 6, 7, 9 and 10. However, the expression pattern might vary, depending if they are isolated from lymphatic tissue or peripheral blood and on the B-cell subset. TLR9 expression and responsiveness is increased in B-cells isolated from tonsils when compared to those isolated from peripheral blood (111). Moreover, naïve B-cells only express low levels of TLRs and, thus, poorly respond to TLR stimulation, whereas memory B-cells proliferate and differentiate into plasma cells upon TLR stimulations (112–115).

Upon ligand binding, the activated TLRs recruit Toll/interleukin-1 receptor (TIR) homology domain containing adaptor proteins to start a phosphorylation cascade (116). Interestingly, different TLRs are able to trigger different signaling pathways depending on which adaptor protein is recruited (100, 117). Although the TLR engagement is multifaceted and implies additional factors besides the type of adapter protein, the signaling pathways can be separated in two groups: 1 - the Myeloid differentiation primary response gene 88 (MyD88)-dependent pathway that leads to the production of pro-inflammatory cytokines with

activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK); 2 - the MyD88-independent pathway associated with the TIR-domain-containing adapter-inducing interferon-β (TRIF) and translocation associated membrane protein (TRAM) adaptors, which activate TRAF family member-associated NF-kappa-B activator (TANK)-binding kinase 1 (TBK1) and IκB kinase-ε (IKKε) that leads to the induction of type I IFN and IFN-inducible genes (118). Furthermore, TLR ligands induce cell-type-specific responses, but the involved mechanisms to explain how this is achieved are only partly revealed (119).

Preformed inactive TLR9 homodimers, which we focused on in the present project, are initially present in the endoplasmatic reticulum (ER) of B-cells and dendritic cells. The TIR domains stay separated in order to avoid the spontaneous recruitment of MyD88 (120). Upon CpG oligonucleotide (ODN) uptake, TLR9 translocates to the same endo-lysosomal compartments, which results in ligand binding and signal activation (121, 122). The binding of CpG ligand leads to a conformational change that brings the cytoplasmic TIR domains together (123). Subsequently, the TIR domains associate with the TIR domain-containing adaptor MyD88 (123). The latter recruits the interleukin-1 receptor-associated kinase (IRAK) 4 to TLR9 through interaction of the death domains of both molecules. IRAK-1 is activated by phosphorylation and associates with the TNF receptor associated factor (TRAF) 6, thereby activating the IκB kinase (IKK) complex, leading to activation of mitogen-activated protein (MAP) kinases (JNK, p38, MAPK) and of nuclear factor kappa B (NF-κB). NF-κB promotes the transcription of genes involved in the cellular activation, proliferation and in the production of pro-inflammatory cytokines (124) (**Fig. 1**). Although human B-cells are generally considered poor cytokine producers, stimulation with TLR9 ligands results in the secretion of pro-inflammatory cytokines like IL-1β, IL-2, IL-6, IL-8, and to the release of immune regulatory cytokines that might limit the intensity of the inflammatory response, such as IL10 (115). TLR9 stimulation induces differentiation in the absence of significant proliferation in naïve and transitional human B-cells, whereas a proliferative response is observed in memory B-cells (125). Moreover, TLR triggering of terminally differentiated plasma cells augments Ig production.

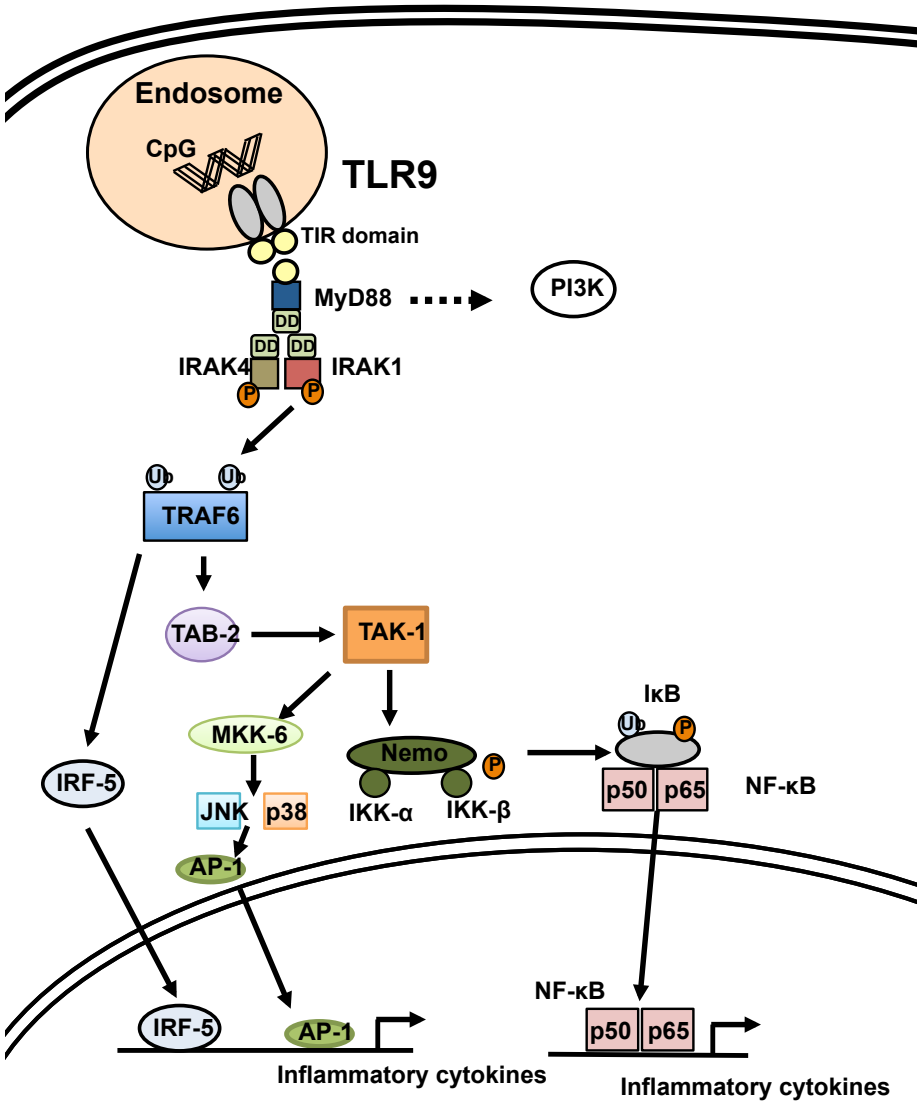

Figure 1: The signaling pathways of endosomal Toll-like receptor 9. The binding of CpG ligand leads to a conformational change that brings the cytoplasmic Toll/interleukin-1 receptor (TIR) domains together. Subsequently, the TIR domains associate with the TIR domain-containing adaptor myeloid differentiation primary response protein 88 (MyD88) followed by the recruitment of IL1-receptor- associated kinase (IRAK) 1 and 4 and tumor-necrosis-receptor-associated-factor 6 (TRAF6). Subsequent activation of Inhibitor of nuclear factor κB-kinase complex (IKK-complex) frees NF-κB from its inhibitor IκB and allows nuclear translocation where expression of pro-inflammatory cytokines is induced. Adapted from Akira & Takeda, Nat Rev Immunol. 2004 (126)

Interestingly, TLR ligands are known to induce cell-type-specific responses, but we only begin to understand how this is achieved. It is still partly unknown weather cell-type specific factors are involved nor if it is the subcellular positioning of certain adaptor molecules that plays a role (119). In the case of TLR9, the secondary and the tertiary DNA structures, and thereby the DNA sequence of potential ligands, have a great influence on the quantity and quality of the conformational changes of TLR9 and on the subsequent receptor

response, as exemplified by the various CpG DNA classes (123). The differential activity of the different CpG classes on immune cells and how the signals are transduced cannot be explained fully to date. Although it is known that different IRFs are activated depending on the type of CpG used, we do not know how the different effects and activated proteins are produced from the same TLR. Moreover, their effects on certain cell types can distinguish the CpG classes. A-class CpGs containing 5' and 3' G-rich stretches induce high levels of type I IFN but show low activation of B cell proliferation (127). B-class CpGs activate B-cells and TLR9-dependent NFκB signaling in recombinant cell lines but show low induction of IFN-α (128). C-class CpGs induce high amounts of IFN-α and activate B cells. The recently discovered new P-Class ODNs show similar but superior properties to C-class ODNs. Even DNA without CpG motifs can act as TLR9 ligands, suggesting that TLR9 recognizes not only the CpG motifs but also other DNA structures (129). In our study we used mainly CpG ODN 2006 a B-class CpG.

Regulation of TLR9 activation by EBV

TLRs activation is linked to antiviral effects through the expression of cytokines and interferons. However, recent data suggest, that several members of the herpesvirus family, such as EBV, hijack the TLR signaling pathways to evade recognition by other immune cells and maintain their latent infection.

Several EBV encoded proteins manipulate the host TLR signaling pathways during latent or lytic phases, and thus, contribute to malignant cell proliferation, survival and viral expansion. EBV's latent membrane protein (LMP) 1 mimics the CD40 receptor signaling, through 3 c-terminal cytoplasmic activation regions (CTAR1, 2 and 3). Each CTAR regions recruits a cellular adaptor protein to initiate a signaling pathway. CTAR1 binds TRAF2 weather CTAR2 binds the TRADD complex both leading to the activation of NF-κB (130, 131). Furthermore, LMP1 induces the expression of IRF4, which competes with IRF5 for the interaction with MyD88 upon TLR-activation. Subsequently, TLR7 and MyD88 expression increases, leading to cellular proliferation (132). EBV's LMP2A has an N-terminal cytoplasmic region containing eight tyrosine residues, which function as the immunoreceptor tyrosine-based activation domain (ITAM) motif (133). The phosphorylated ITAM region recruits Src-type tyrosine kinases, and this ITAM signalosome-mediated ERK-activation also

stimulates the c- Jun/AP-1 pathway (134). In addition, non-protein products from EBV, EBER1 and 2 are shown to affect host cell signaling. EBER1 and 2 are non-translated viral small RNA abundantly expressed in EBV latently infected cells. EBER activate the NF-κB and IRF3 pathways through association with RIG-I (135, 136). Besides latent EBV gene products, also lytic EBV genes impact the signaling induced downstream of TLRs. ZEBRA, the protein encoded by the immediate-early gene *BZLF1* of EBV is on the one hand inducing NF-κB translocation into the nucleus (137), but is also able to inhibit binding of NF-κB to responsive promoters (138). All of these cellular signaling molecules and transcription factors are also activated downstream of TLR activation. This shows that EBV by itself modulates the innate immune system thereby preventing immune recognition of EBV-infected cells.

Furthermore, TLR9 signaling contributes to shape the EBV viral gene expression and, thus, the balance between lytic and latent cycles. Earlier studies in our lab found that stimulation of BL cells with synthetic CpG ODN 2006 ligands of TLR9 suppresses EBV lytic reactivation in Vitro (48, 139). Transfection of p65, a subunit of NF-κB, was shown to inhibit the lytic replication of gamma-herpesviruses (140). However in the case of EBV, our group showed NF-κB not to be fully responsible for the TLR9-induced inhibition of lytic reactivation in BL cells suggesting an alternative mechanism to play a role (48).

Finally, TLR9 activation turns out to be a double-edged sword: on one hand it induces and shapes the adaptive immune response and on the other hand it facilitates the long-term infection of EBV and the lymphomagenesis through induction of the infected host B-cell's proliferation and survival. Thus, in the situation of EBV and *P. falciparum* co-infections, TLR stimulation might influence and disturb the balance between the infection and the immune control in asymptomatic long-term latency and might offer an explanation to the ontology of EBV-associated Burkitt's lymphoma in immunocompetent patients.

Epigenetics

Overview of the different epigenetic mechanisms

The term epigenetic, according to Ptashne's definition, describes "a change in the state of expression of a gene that does not involve a mutation, but that is nevertheless inherited in the absence of the signal or event that initiated the change" (141, 142).

The most important epigenetic mechanisms include DNA methylation, histone post-translational modification and non-coding RNAs. These factors, influenced by many environmental agents, including: - heavy metals, - pesticides, - diesel exhaust, - tobacco smoke, - poly- cyclic aromatic hydrocarbons, - hormones, - radioactivity, - viruses, - bacteria, and - basic nutrients, alter how genes are expressed (143). Epigenetic changes, although reversible, might persist through cell divisions for the duration of the cell's life, and may last for multiple generations without altering the underlying DNA sequence (144).

DNA methylation occurs mostly in CpG dinucleotides, at the 5th carbon of the cytosine residue and is associated with transcriptional silencing. Methylation plays also an important role in processes like imprinting, X-chromosome inactivation, silencing of repetitive and centromeric sequences (145).

The genetic material of eukaryotic cells is packaged in the nucleus in the form of chromatin. Chromatin is made by octamers of four histone proteins – H2A, H2B, H3 and H4 – around which the DNA is wound almost twice, in a unit called nucleosome (146). Histone post-translational modifications (PTM) alter the chromatin structure by the covalent addition or removal of functional groups like: -acetyl, -methyl, -phosphoryl, or -sumoyl, on the histones tails that protrude from the nucleosome and that are freely accessible to enzymes - the so called writers that create these covalent modifications (eg, DNA and histone methyltranferases, histone acetyl- transferases, etc.). These modifications influence directly the histone-histone and the histone-DNA interactions, thereby altering the DNA stability and accessibility to transcription factors and to the transcription machinery (145). In general, tightly folded chromatin (heterochromatin) tends to be inactive, while more open chromatin (euchromatin) is functional. Furthermore, protein effectors, also called readers, can recognize specifically and bind to the histone PTMs through specific protein domains (eg, bromodomain-containing proteins). The readers are the major players that govern dynamic changes in chromatin structure. On the basis of developmental or environmental stimuli, they recruit various components of the nuclear signaling network to the chromatin, mediating *via* chromatin remodeling fundamental processes such as gene transcription, DNA replication and recombination, DNA damage response. The readers bind specifically to certain histone PTMs, and the misreading of the histone PTM code has been shown to cause various diseases (147, 148). The histone PTMs are reversible and can be removed by enzymes called erasers. The gene expression patterns can also be affected by non-covalent histone modifications, including ATP-dependent chromatin remodeling and introduction of histone variants (145).

Finally, non-coding RNAs, genomic imprinting and gene silencing are other prominent examples of epigenetic mechanisms that are essential for cell fate decisions (149).

Epigenetic regulation of *BZLF1* promoter and viral genome

One of the hallmarks of EBV, the capacity to establish latent infection in the host, is regulated by diverse epigenetic mechanisms.

The EBV viral capsid contains a linear double stranded DNA in an epigenetically naïve state. One of the first steps, upon infection of a host cell, is the circularization of the viral DNA in order to protect the DNA ends from degradation and avoid the induction of a DNA damage response (150). The circular DNA associates with histones and other cellular DNA-associated proteins to form nucleosomes (151) and the CpG dinucleotide become methylated (152). Thus, the viral DNA acquires all the features of cellular chromatin.

The four types of global epigenetic modification known to repress EBV's lytic viral genes during latency are: - histone modifications, - nucleosomal density and compaction of DNA, - DNA methylation, and - chromatin architecture. These modifications repress the expression of the viral lytic genes and tightly regulate the viral latent gene expression program.

Although silencing of gene expression is essential for EBV to escape immune recognition during the latent phase, the advantage of epigenetic silencing is its efficient and fast reversion. Upon EBV lytic induction, a signaling cascade induces the expression of the immediate early viral transcription factor *BZLF1*, which in turn activates the lytic transcription cascade that, within few hours, leads to viral particle synthesis. *BZLF1* preferentially binds to a subset of CpG-methylated Zebra responsive element (ZRE) motifs. Due that the latent EBV genome, and especially the ZRE-containing lytic promoters, heavily features CpG DNA methylation, *BZLF1* can disrupt the latency efficiently (76, 77, 153).

The expression of *BZLF1* is restricted by repressive factors including Jun dimerization protein 2 (JDP2) (154), zinc finger E-box binding factor (ZEB) (89), Yin Yang 1 (YY1) (155), and an unidentified repressor that binds to the ZIIR motif (156). In addition to this, like the other viral genes, *BZLF1* expression is regulated by epigenetic modifications. While other EBV promoters are heavily methylated, there are very few CpG motifs, methylated or not, in the *Zp* promoter region of *BZLF1* (157, 158). One possible explanation is that the silencing of

BLZF1 by CpG methylation would be too strong for a quick response. *BZLF1* would not be expressed easily enough upon stimulation. Therefore, *Zp* is primarily silenced and regulated by histone modifications. Indeed, treatment of latently infected B-cells with histone deacetylase (HDAC) inhibitors, like sodium butyrate or trichostatin A (TSA) can reactivate the viral lytic phase in certain cells lines like, for example, the Akata BL cells (86, 159).

We and others used chromatin immunoprecipitation assays to demonstrated that suppressive histone markers, including tri-methylation of histone H3 on lysine 27 (H3K27me3), H3K9me2/3 and H4K20me3, are present in the *Zp* region of latent cells and that strong histone acetylation, phosphorylation, and H3K4me3 markers correlate with reactivation of the virus (86, 88, 160, 161).

H3K27me3 is a characteristic marker of facultative heterochromatin, a reversible form of heterochromatin where the expression of a wide variety of genes is silenced by histone modifications (162). This type of heterochromatin can be reversed by specific signals, to become transcriptionally active, unlike constitutive heterochromatin.

Thus, through chromatinization of its DNA genome, EBV mimics the host's gene silencing, using epigenetic mechanisms to shut down its genome and hide from the immune recognition. Moreover, EBV exploits this reversible gene silencing to quickly and efficiently overcome the repression of its lytic genes within few hours after the induction of its lytic cycle, leading to de novo virus synthesis.

TLR9 regulation of histone modification and gene expression

As we have seen, TLRs recognize PAMPS or DAMPs and activate specific signaling cascades that lead to the expression of proinflammatory cytokines, chemokines and antiviral interferons. The TLR induced response, at the heart of every innate immune response, is dynamically controlled and cell type-specific. Of course, transcription factors like NF-κB, AP1, IRFs and others are crucial to transduce the signal from the TLRs to the promoters and the molecular signaling events, leading from the TLRs to the promoter regions of inflammatory response genes have been described in great detail. Far less is known about the role epigenetics plays in shaping the transcriptional responses downstream of the TLRs, as well as other innate immune receptor systems. However, epigenetic factors/chromatin-based mechanisms have proved to be critical for context-specific gene expression in diverse innate

immune cell types (163, 164). Recent publications have shown that TLR-induced epigenetic changes constitute a critical new mechanism in both the positive and negative regulation of TLR-induced genes. TLR-induced chromatin remodeling on the IL12p40 promoter has been described, indicating that chromatin remodeling is an additional level of TLR signaling specificity (165, 166). Foster et al found that individual host gene promoters can be targeted and modulated in its histone architecture after TLR engagement (164). Repeated triggering of TLRs was shown to repress activation of selected TLR-responsive promoters (TLR tolerance), while other TLR-responsive promoters were not affected (164). Moreover, TLRs also directly interfere with histone structure of pathogen gene promoters. Our group showed that TLR9 induction is changing the acetylation and phosphorylation states of histones on the *BZLF1* lytic gene promoter of EBV leading to suppression in lytic gene expression (48). Thus, TLRs can interfere with lytic activation of a chronic pathogen like EBV residing in its latent form *via* alteration of histones on the viral gene promoter.

Thus, one significant question to be answered is the molecular means by which general chromatin writers are targeted to the promoters of individual host and viral genes upon TLR9 triggering. No study has identified the exact mechanism by which TLR9 stimulation induces changes in the histone architecture. TLR engagement strongly induces distinct sets of MAP kinase kinases (MKKs/MEKs) as well as MAPK phosphatases, e.g. DUSP1 (MAPK phosphatase 1) (167). In turn, these enzymes are able to differentially regulate the activity of the AGC kinase family members, e.g., MSK1/2 and RSK2, which are known to affect histone phosphorylation (167, 168). In the context of histone acetylation, TLR9 might affect the recruitment of co-activators, which have histone deacetylase (HDAC) or histone acetyl-transferase (HAT) activities (like p300/CBP), leading to chromatin remodeling on host and viral promoters and subsequent changes in gene expression. Importantly, MSKs, HDACs and HATs are known to modify a range of other non-histone proteins including transcription factors such as CREB, ATF-1, and NF-κB.

SUBJECT OF INVESTIGATION

The main topic of my thesis work was the mechanisms by which innate immune stimulation influences EBV lytic reactivation in BL cells. The following topics were subject of investigation:

1) **From TLR9 stimulation to EBV lytic inhibition. A detailed analysis of the TLR9 signaling pathway in BL cells and how it impacts *BZLF1* expression. (Manuscript 1)**

In this manuscript I investigated the signaling pathway linking TLR9 to the *Zp* promoter. Previous published results showed that neither NF-kB nor other known downstream components of the canonical TLR9 signaling pathway are keys for the TLR9-mediated suppression of lytic EBV, suggesting an alternative pathway to play a role. First, in order to direct the research, we tested if the TLR9-induced lytic inhibition requires a first round of *de novo* protein synthesis. Then, using siRNA and CRISPR Cas9 silencing technologies we targeted MyD88, IRAK4 and IRAK1 downstream of TLR9. For this project I performed all the CRISPR/Cas9 plasmids cloning, controls, WB and activity assays and I designed the experiments and supervised Jeannine Marty, a master student, for the protein synthesis and siRNA experiments. For the supplementary WB asked by the Journal of Virology reviewers, I stimulated and lysed the cells and Vanessa Mordasini performed the WB.

2) **From TLR9 stimulation to EBV lytic inhibition. A detailed analysis of the *Zp* lytic promoter upon TLR9 and/or BCR stimulation in reporter cells lines. (Manuscript 2)**

To characterize the effect of TLR9 on the *Zp* promoter sequence in detail, we evaluated the TLR9-induced suppression for different *Zp* regions coupled to a luciferase gene in a reporter system. Unexpectedly, TLR9 triggering did not affect the activation of *Zp* reporter constructs, independently of the length or region of the *Zp* promoter used on the reporter. Thus, we hypothesized that TLR9-induced histone modifications observed in genomic EBV upon lytic reactivation were not found in episomal reporter constructs. I completed all the figures with experiments using the pHEBo-2.3 kb *Zp* luciferase reporter plasmid and contributed to the second part of this manuscript, investigating the reason for the

different regulation of the reporter constructs, performing all the chromatin immunoprecipitation assays for the genomic EBV and the different reporter construct.

3) **How does the TLR9 polymorphism influence the effect of CpG agonists on BL cells? (Manuscript 3)**

Here, we used BL-cell lines to model direct effects of TLR9 stimulation on malignant cells, investigate the influence of EBV, and assess the impact of TLR9 SNPs, which we found in primary BL samples or in healthy primary cells. My contribution to this paper was the investigation of the cell death mechanism by measuring caspase activity and PARP cleavage and performing the assays with caspase inhibitors.

RESULTS

Manuscript I: IRAK4 is essential for TLR9-induced suppression of Epstein-Barr virus *BZLF1* transcription in Akata Burkitt's lymphoma cells

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Manuscript submitted for publication

Abstract

Latent Epstein-Barr virus (EBV) infection is associated with endemic Burkitt's lymphoma (eBL) that is epidemiologically linked to chronic infection with the malaria agent *Plasmodium falciparum*. Lytic reactivation of EBV eventually leads to the death of the host cell. Previously, we showed that activation of the innate immunity signaling via Toll-like receptor (TLR) 9 by hemozoin, a pigment of *P. falciparum,* is critical in inhibiting EBV lytic reactivation and thereby causing reinforcement of its latency in eBL cells. Here, we show that EBV lytic repression is reinforced by TLR9 engagement via a mechanism that is largely protein synthesis independent and that two TLR9 downstream signaling molecules, MyD88 and IRAK4, exhibit central roles. Counteracting signaling of these molecules could allow lytic reactivation and thus provide novel treatment targets to combat eBL.

For detailed information see attached manuscript 1. This manuscript is the main part of my work and thus will be the essential part in the discussion and outlook section.

Complementary Data and Results to Manuscript I

A residual IRAK4 activity is sufficient to inhibit viral lytic reactivation in Burkitt's lymphoma cells after TLR9 triggering

As shown in the manuscript, the inactivation of TLR9, MyD88 or IRAK4 in CRIPSR/Cas9 single cell clones rescued the EBV lytic reactivation upon treatment of BL cells with ODN CpG 2006 prior to BCR cross-linking. To know if residual TLR9 activity impacts on EBV lytic reactivation, we stably transfected cells with shRNA against IRAK4 (shIRAK4). The percentage of Akata cells containing the plasmid was determined for each cell line produced, by flow cytometry for GFP-expressing cells. IRAK4 silencing was verified at the mRNA and protein levels by RT-qPCR and Western Blot, respectively. As further controls, and to verify that the expression of shRNA for IRAK4 does not change the expression level of the receptors to be stimulated, the IgG protein expression on the cells surface and the TLR9 mRNA level were measured by flow cytometry and RT-qPCR, respectively. Finally, the *IL-10* mRNA level was used as readout for the TLR9 pathway activity. The degradation of IRAK1 upon recruitment to the TLR9-MyD88-IRAK4 complex and phosphorylation by IRAK4 (169, 170) was used as a secondary readout for TLR9 pathway activity.

More than 98% of the independently produced $Akata_{shIRAK4\_1}$, $Akata_{shIRAK4\_2}$, $Akata_{shIRAK4\_3}$, $Akata_{shIRAK4\_4}$ and $Akata_{shIRAK4\_1}$ cell pools, transfected with shIRAK4 or shControl plasmids, expressed GFP as determined by flow cytometry (data not shown) and therefore contained the transfected plasmid. We measured a reduction of *IRAK4* mRNA of 20% in $Akata_{shIRAK4\_1}$, of 55% in $Akata_{shIRAK4\_2}$, of 52% in $Akata_{shIRAK4\_4}$, and of 40% in $Akata_{shIRAK4\_3}$ (**Fig. 2a**). This reduction was also detectable at the protein level, and ranged from a 50% IRAK4 expression reduction in $Akata_{shIRAK4\_1}$ to 80% in $Akata_{shIRAK4\_2}$ and $Akata_{shIRAK4\_3}$ and was below detection in $Akata_{shIRAK4\_4}$ (**Fig. 2b**). An Akata cell line expressing a dominant negative form of MyD88 (48) ($Akata_{MyD88\text{-}DN}$) was used as a positive control for inactivation of the TLR9 pathway in the different experiments. The level of IRAK4 mRNA remained stable but a 70% protein expression reduction was measured in the $Akata_{MyD88\ DN}$ cells when compared to the $Akata_{shcontrol}$ cells. The reduced IRAK4 expression in $Akata_{MyD88\ DN}$ cells could be explained by decreased IRAK4 protein stability caused by the lack of myddosome complex formation. Indeed, upon ODN CpG 2006 stimulation of TLR9,

MyD88 binds to TLR9 dimers and initiates the formation of a protein complex with IRAK4, IRAK1 and other proteins called myddosome (171).

Surface IgG protein (**Fig. 2c**) and TLR9 mRNA (**Fig. 2d**) expression levels were not affected by the IRAK4 down regulation in the 4 independent shIRAK4 clones tested, when compared to the cells transfected with a control plasmid ($Akata_{shcontrol}$).

Finally, *IL-10* mRNA expression after ODN CpG 2006 treatment, measured as a readout for TLR9 pathway activity, was reduced about 2-fold in $Akata_{shIRAK4\_1}$, and about 3-fold in $Akata_{shIRAK4\_2}$, $Akata_{shIRAK4\_3}$, and $Akata_{shIRAK4\_4}$ when compared to *IL-10* mRNA expression in $Akata_{shcontrol}$ cells (**Fig. 2e**). IRAK1 protein expression, measured as a second readout for TLR9 pathway activity, was decreased to about 70% after 8h stimulation of $Akata_{shcontrol}$ cells with ODN CpG 2006, and, importantly, was decreased to about 20% after ODN CpG 2006 stimulation of $Akata_{MyD88\text{-}DN}$ control cells. This is consistent with a reduced signaling activity of the TLR9 pathway (**Fig. 2f**). $Akata_{shIRAK4\_1}$ and $Akata_{shIRAK4\_3}$ showed a 50% decrease of IRAK1 protein after ODN CpG 2006 treatment, whereas the $Akata_{shIRAK4\_2}$ and $Akata_{shIRAK4\_4}$ showed no significant changes. Taken together, these results show that the *IRAK4* mRNA and protein levels could be decreased by the stable transfection of shRNA-IRAK4 and result in a reduced, but not completely abrogated, TLR9 pathway activity.

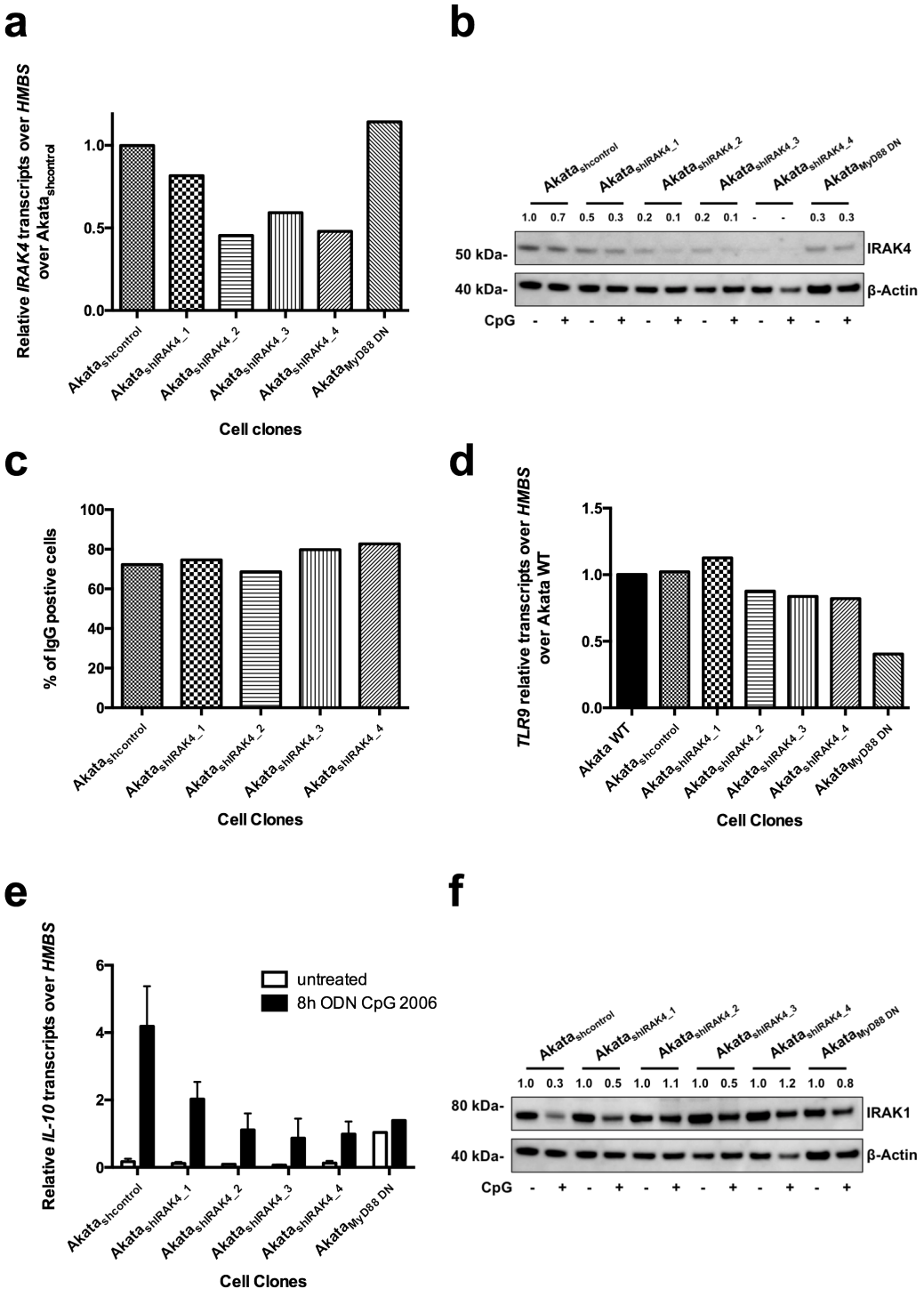

Figure 2: Silencing of IRAK4 by shRNA does not completely abrogate TLR9 signaling activity. Independently produced Akata cell pools, stably transfected with shIRAK4 or shControl, were named $Akata_{shIRAK4\_1,\ 2,\ 3\ and\ 4}$ and $Akata_{shcontrol}$. Akata cell line expressing a dominant negative form of MyD88 ($Akata_{MyD88\ DN}$) was used as further control. (**a**) *IRAK4* relative mRNA expression level over *HMBS* over $Akata_{shcontrol}$ was measured by RT-qPCR (**b**) IRAK4 protein level was measured by Western Blot before and after ODN CpG 2006 treatment for 8h. IRAK4 values were calculated relatively to β-actin and normalized to the untreated sample of $Akata_{shcontrol}$. (**c**) Percentage of IgG positive cells tested by flow cytometry. (**d**) *TLR9* relative mRNA expression level over to *HMBS* normalized to Akata WT cells measured by RT-qPCR. (**e**) *IL-10* mRNA expression level relative to *HMBS* measured by RT-qPCR after 8h ODN CpG 2006 treatment. n= 3. (**f**) IRAK1 protein depletion measured

by Western Blot 8h after ODN CpG 2006 treatment. IRAK1 values were calculated relatively to the β-actin level of the same sample. Untreated samples were set to one and ODN CpG 2006 treated samples calculated relatively to it.

To know if the residual TLR9 activity is sufficient to inhibit EBV lytic reactivation or if the *BZLF1* expression can be rescued by the IRAK4 protein reduction, Akata WT cells stably transfected with shRNA against IRAK4 were treated for 2h with ODN CpG 2006 followed by treatment with anti-IgG for additional 6h. Interestingly, independently of the IRAK4 protein level and TLR9 pathway activity, the activation of the TLR9 pathway by ODN CpG 2006 inhibited the relative *BZLF1* transcripts over *HMBS* over unstimulated cells induced by anti-IgG treatment by about 72.1 % (p=0.0182) in $Akata_{shIRAK4\_1}$ cells, 83.2 % (p=0.004) in $Akata_{shIRAK4\_2}$ cells, 82.3 % (p=0.0112) in $Akata_{shIRAK4\_3}$ cells and 77.2 % (p= 0.0007) in $Akata_{shIRAK4\_4}$ cells (**Fig. 3**). For comparison, the activation of TLR9 in $Akata_{shcontrol}$ cells reduced about 86.1 % (p=0.0228) the relative *BZLF1* transcripts expression induced by anti-IgG treatment. The $Akata_{MyD88\ dn}$ control cells, on the other hand, showed a rescue of the *BZLF1* expression after ODN CpG 2006 and anti-IgG treatment (**Fig. 3**).

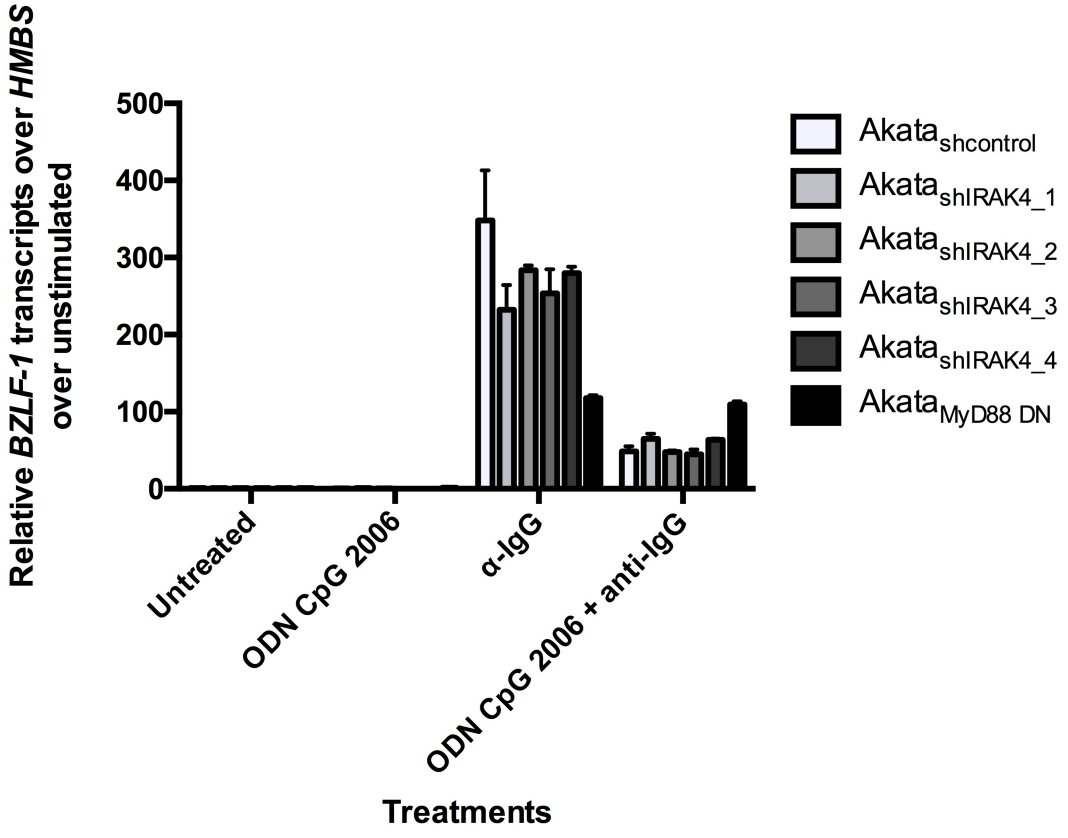

Figure 3: TLR9 signaling activity of 30% or less is sufficient to inhibit *BZLF1* expression after activation through ODN CpG 2006. *BZLF1* mRNA expression level relative to *HMBS* was measured by RT-qPCR after ODN CpG 2006 pre-treatment for 2h followed by 6h incubation of stably transfected $Akata_{shIRAK4}$ cells with anti-IgG. Untreated *BZLF1* values were set to one and treated samples calculated relatively to it. One representative experiment out of three is shown.

Taken together, these results indicated that reduction of the TLR9 signaling activity, measured by the IL-10 mRNA increase and IRAK1 protein depletion after ODN CpG 2006 treatment, does not affect the TLR9-induced inhibition of *BZLF1* expression upon BCR cross-linking. Moreover, these results suggested that residual IRAK4 kinase activity is sufficient to amplify the TLR9 signal leading to a complete inhibition of EBV lytic reactivation.

Manuscript II: TLR9 induction suppresses Epstein-Barr virus lytic reactivation in Burkitt's lymphoma cells by modifying histones structure on the genomic *Zp* promoter but not on reporter systems

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Manuscript under preparation

Abstract

Background: The triggering of Toll-like receptor 9 (TLR9) results in the suppression of EBV's master regulatory lytic gene *BZLF1*. The reduction of EBV lytic gene expression promotes viral latency in the cells and latent EBV, in turn, might enhance the possibility for cell transformation. Lytic reactivation of EBV is associated with modification of chromatin structure on the *BZLF1* promoter *Zp*.

Results: To characterize the effect of TLR9 on the *BZLF1* promoter *Zp* in more detail, we evaluated the TLR9-induced suppression of lytic EBV for different *Zp* regions coupled to a luciferase gene in a reporter system. Unexpectedly, TLR9 triggering inhibited *BZLF1* expression in BCR-induced Akata Burkitt's lymphoma cells latently infected with EBV, but did not affect the activation of *Zp* reporter constructs. TLR9-induced histone modifications observed in genomic EBV upon lytic reactivation were not found in episomal reporter constructs.

Conclusions: The underlying mechanism of the differential effect of TLR9 signaling might mirror the distinct chromatin structure of the transfected *Zp* reporter construct compared to EBV's viral genome. This reporter system based research does not reflect the viral reality and other strategies should be considered to study EBV's lytic reactivation in order to use it for cancer cell-tailored therapeutic approach.

For detailed information see attached manuscript 2.

Manuscript III: TLR9 agonists induced cell death in Burkitt's lymphoma cells is variable and influenced by TLR9 polymorphism

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Abstract

Toll-like receptor 9 (TLR9) triggering is a promising novel strategy to combat cancer as it induces innate and adaptive immunity responses. B-cell lymphoma is unique in this context as tumor cells express TLR9 and may harbor latent Epstein-Barr virus (EBV), a gamma-herpesvirus with remarkable oncogenic potential when latent. Latent EBV may be promoted by TLR9 triggering via suppression of lytic EBV. Here, we elaborated an initial assessment of the impact of TLR9 triggering on EBV-positive and EBV-negative B-cell lymphoma using Burkitt's lymphoma (BL) cell lines as an in vitro model. We show that, independent of the presence of EBV, the TLR9 ligand oligodeoxynucleotide (ODN) CpG-2006 may or may not induce caspase-dependent cell death in BL cells. Moreover, ODN CpG-2006-induced cell death responses of BL cells were associated with TLR9 single-nucleotide polymorphisms (SNPs) rs5743836 or rs352140, which we detected in primary BL tumors and in peripheral blood from healthy individuals at similar frequencies. Thus, our findings suggest that the effect of TLR9 agonists on BL cells should be tested in vitro before installment of therapy and TLR9 SNPs in BL patients should be determined as potential biological markers for the therapeutic response to treatment targeting innate immunity.

For detailed information see attached manuscript 3.

GENERAL DISCUSSION AND OUTLOOK

Our group used eBL cell lines as instrument to investigate the molecular mechanisms regulating the inhibition of lytic EBV. eBL is strongly associated to the co-infection with P. falciparum and EBV but develops also in HIV patients (30% of which are EBV positive). Interestingly, the infections by P. falciparum, HIV or primary EBV infection induce a great GC expansion. According to genetic, phenotypic and transcriptional analysis the origin of BL are GC B-cells. Although the mutation point is different, the eBL as well as the AIDS-associated BL and the sBL are characterized by a chromosomal translocation between the c-myc oncogene and one of the immunoglobulin loci, which leads to a constitutive expression of c-myc and to the uncontrolled growth of the cell. The *c-myc* translocation might thus by mediated through the expression of AID, which is highly expressed in the GC as it is responsible for the somatic hypermutation and class switch recombination of immunoglobulin genes as they undergo affinity maturation in the GC (31–33). Thus, factors like Malaria, EBV or HIV infection, inducing a GC expansion, increase the probability of c-myc translocation. Normally, the uncontrolled expression of c-myc as observed in eBL cell should lead to the apoptosis of the cell, but there are studies suggesting that the EBV infection of the cells provides a resistance to this apoptosis (36). The understanding of c-myc and EBV influence in eBL development has progressed in the last years, while the role of P. falciparum remains poorly understood.

Our group could previously show that hemozoin, a disposal product formed from the digestion of blood by P. falciparum, suppresses through the activation of TLR9, the lytic reactivation of EBV in BL cells by affecting *BZLF1* promoter histone sites (48). Here we aimed to characterize more in detail the TLR9-induced inhibiting mechanisms. In this work, we used ODN CpG 2006 as a TLR9 rather than hemozoin, as a TLR9 agonist. First, to direct our investigation, we tackled the question whether protein synthesis is decisive in EBV's reactivation process upon TLR9 activation of host cells. Using translation inhibitors, we could provide evidence that TLR9-mediated inhibition of anti-IgG triggered BZLF1 expression does not require de novo protein synthesis. Protein translation inhibitors, as expected, abrogated the positive feedback loop that ZEBRA exerts on its own *Zp* promoter to amplify the lytic reactivation signal upon BCR cross-linking. But interestingly, TLR9 stimulation inhibited *BZLF1* mRNA expression to a similar degree, whether protein synthesis was inhibited or not. This strongly suggests that the regulation of EBV's lytic reactivation upon TLR9 stimulation

with ODN CpG 2006 is, to a large extend, protein synthesis independent and suggests post-translational modifications to be responsible for EBV lytic gene repression. Moreover, TLR9-induced cytokines like IL-10, IL-12 or IFNγ can be excluded to play a rule in the lytic inhibition in our system (139). The question weather TLR9 activation also affects ZEBRA's activity via sumoylation (172) or other post-translation modifications remains open. Finally, even if, due to incomplete translation inhibition, we cannot completely exclude the existence of a newly synthesized unknown repressor factor(s), our results strongly indicate that EBV reinforces its latency by hijacking the TLR9-induced post-translational modifications to repress its lytic genes expression (Manuscript 1). Then, based on previous observations which stipulates that the suppression of lytic EBV treatment with ODN CpG 2006 is mediated through TLR9 but is independent of TLR9-elicited NF-kB activity as well as PI3K, ERK, JNK or p38, other known TLR9 signaling components (48) we inactivated *TLR9*, *MyD88*, or *IRAK4* genes in single cell clones using the CRISPR/Cas9 methodology. We unequivocally and unprecedentedly show that MyD88 and IRAK4 are essential for the TLR9-dependent inhibition of EBV lytic reactivation upon BCR cross-linking (Manuscript 1). By contrast, silencing of IRAK4 with shRNA showed that a 70% decrease of *IRAK4* mRNA and protein, which is sufficient to reduce the TLR9 pathway activity, was not sufficient to abolish the TLR9-blockade of EBV lytic reactivation. This suggests that a residual IRAK4 and thereby a low TLR9 signaling pathway activity, after stimulation with ODN CpG 2006, are sufficient to maintain EBV in a latent state upon BCR cross-linking with anti-IgG. It is commonly accepted that protein kinase activity is particularly difficult to block by silencing, considering that the residual kinase activity might be amplified. In fact, IRAK4 recruits and activates IRAK1 (169, 170), which, upon activation by IRAK4, is proposed to act in a positive feedback loop to activate other IRAK1 molecules and members of the IRAK family (170). This suggests a role for IRAK1 as an amplifier of the TLR9 signaling pathway, which would override the silencing of IRAK4 in TLR9 pathway activity. This would imply that IRAK4 is not an ideal target for intervention to support EBV lytic reactivation upon BCR cross-linking in TLR9 stimulated BL cells. Indeed, only a 100% inactivation of the IRAK4 protein, obtained by mutation of the gene using the CRISPR/Cas9 method, was able to rescue the TLR9-induced EBV lytic inhibition. Intriguingly, using the CRISPR/Cas9 method, we failed to establish IRAK1 mutated BL cell clones retaining EBV genomes. This preliminary finding suggests that IRAK1 could play a central role in steering EBV persistence. EBV nuclear protein EBNA1 binds to the origin of viral replication and is essential to mediate replication and partitioning of its episome during the division of the host cell. EBNA1 function is

regulated by the phosphorylation of ten specific sites (173). The kinases responsible for EBNA1 phosphorylation have not been identified, but IRAK1 could be a potential candidate. Thus, one hypothesis for the loss of EBV in the IRAK1-mutated clones is that EBNA1's function is altered by the inactivation of IRAK1, which affects the replication and partitioning of EBV's genomic DNA in dividing cells leading to the dilution of the virus in the daughter cells and finally to its loss. However, it is important to consider that this result has only been observed only in a limited number of clones, mostly transfected with the IRAK1-b CRISPR/Cas9 plasmid, the most efficient one, in three independent single cell cloning round. The potential importance of IRAK1 activity for EBNA1 function requires the analysis of more clones transfected with different IRAK1- CRISPR/Cas 9 plasmids as well as further investigations (Manuscript 1).

The *BZLF1* minimal promoter region *Zp-221* is known to harbor the elements for maintaining low basal activity and for the transcriptional activation by agents inducing lytic EBV infection (174, 175). Notably, upstream of -221 bp several negative elements have been identified (155, 176). Nevertheless, the *Zp* promoter regions affected upon TLR9 triggering remain unknown. Using different *Zp* regions coupled to a luciferase gene in a reporter system, we could show that, unexpectedly, TLR9 triggering inhibited *BZLF1* expression in BCR-induced Akata Burkitt's lymphoma cells latently infected with EBV, but did not affect the activation of *Zp* reporter constructs. Lytic reactivation of EBV is associated with modification of chromatin structure of the *BZLF1* promoter *Zp*. Using ChIP assays, we observed that TLR9-induced inhibition of histone H3K9 acetylation at the full length genomic *Zp* region did not take place on the different *Zp* reporter constructs. This indicated that TLR9 triggering has a different effect on chromatin structure associated with genomic EBV than that associated with reporter plasmids. This, in turn, strongly suggests that the suppressive effect of TLR9 signaling on *Zp* promoter activation is linked to a specific chromatin conformation associated with genomic EBV. Our findings further support the importance of chromatin reorganization in the regulation of transcription of *BZLF1* and therefore in the balance between EBV latency and lytic reactivation in cancer cells. We showed that a reporter system based research does not reflect the full-length genomic viral context. Thus, one must consider other strategies for the further elucidation of chromatin reorganization in the regulation of transcription of *BZLF1* and the study *Zp* regulatory elements. Moreover, precaution should be taken when interpreting reporter system based results (Manuscript 2).

Synthetic TLR9 agonists are potential anti-cancer agents. B-cell activation can eventually lead to activation-induced cell death of cancer cells and therefore support anti-cancer treatment. Therefore, we used BL-cell lines as an in vitro model for B-cell tumor to measure the direct effects of TLR9 agonists on malignant cells, investigate the influence of EBV, and assess the impact of TLR9 SNPs, which we found in primary BL samples or in healthy primary cells. We found that treatment with TLR9 ligands induces distinct cytokine expression and cell death responses in distinct BL cells. TLR9-induced apoptosis could be suppressed by pan caspase inhibitors, was not dependent on the presence or absence of EBV in the tumor cells and was associated with SNPs in the *TLR9* gene. Thus, therapeutic TLR9 triggering appears to be a double-edge sword that may induce apoptosis, or enhance lymphoproliferation. Our findings suggest that the effect of TLR9 agonists on BL cells should be tested in vitro before installment of therapy and that TLR9 SNPs in BL patients should be evaluated as potential biological markers for the response to treatment targeting innate immunity (manuscript 3).

Finally, the presence of latent EBV in cancer cells and its capacity to kill its host cell upon lytic reactivation makes EBV an interesting target for the development of oncolytic therapies with the aim to kill specifically the EBV infected cancer cells and to spare the healthy bystander cells. In vitro, this can be initiated easily by a variety of agents and by cross-linking the immunoglobulin of the B-cell receptor on EBV-harboring cells but in vivo the EBV-lytic reactivation is more challenging. In transformed endemic BL cells, after c-myc translocation, the death of the tumor cells by a switch to lytic EBV might be blocked because of P. falciparum induced TLR9 activation, leading to an increased survival of the EBV-infected malignant cells. Thus, an enhanced understanding of the regulation of EBV lytic reactivation by cellular signaling pathways, including TLR9 signaling, will allow optimizing therapeutic strategies based on manipulation of chromatin remodeling.

REFERENCES

1. **Taylor GS**, **Blackbourn DJ**. 2011. Infectious agents in human cancers: Lessons in immunity and immunomodulation from gammaherpesviruses EBV and KSHV. Cancer Lett **305**:263–278.
2. **Henle G**, **Henle W**, **Clifford P**, **Diehl V**, **Kafuko GW**, **Kirya BG**, **Klein G**, **Morrow RH**, **Munube GM**, **Pike P**, **Tukei PM**, **Ziegler JL**. 1969. Antibodies to Epstein-Barr virus in Burkitt's lymphoma and control groups. J Natl Cancer Inst **43**:1147–57.
3. **Cohen J**. 2000. Eptein-Barr Virus Infection. N Engl J Med **343**:481–492.
4. **Luzuriaga K**, **Sullivan JL**. 2010. Infectious mononucleosis. N Engl J Med **362**:1993–2000.
5. **Dunmire SK**, **Hogquist KA**, **Balfour HH**. 2015. Infectious Mononucleosis. Curr Top Microbiol Immunol **390**:211–40.
6. **Tsurumi T**, **Fujita M**, **Kudoh A**. 2005. Latent and lytic Epstein-Barr virus replication strategies. Rev Med Virol **15**:3–15.
7. **Burkitt D**. 1958. A sarcoma involving the jaws in african children. Br J Surg **46**:218–223.
8. **EPSTEIN MA**, **ACHONG BG**, **BARR YM**. 1964. VIRUS PARTICLES IN CULTURED LYMPHOBLASTS FROM BURKITT'S LYMPHOMA. Lancet (London, England) **1**:702–3.
9. **Miller 1974 GC-F publication date: A**. 1974. The Oncogenicity of Epstein-Barr Virus. J Infect Dis **130**:187–205.
10. **Damania B**. 2004. Oncogenic gamma-herpesviruses: comparison of viral proteins involved in tumorigenesis. Nat Rev Microbiol **2**:656–68.
11. **Neparidze N**, **Lacy J**, **Haven W**. 2014. Malignancies Associated With Epstein-Barr Virus : Pathobiology , Clinical Features , and Evolving Treatments **12**:358–371.
12. **Raab-Traub N**. 1996. Pathogenesis of Epstein–Barr virus and its associated malignancies. Semin Virol **7**:315–323.
13. **Nilsson K**, **Klein G**, **Henle W**, **Henle G**. 1971. The establishment of lymphoblastoid lines from adult and fetal human lymphoid tissue and its dependence on EBV. Int J cancer **8**:443–50.

14. **Raab-Traub N**. 2007. EBV-induced oncogenesisHuman Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge: Cambridge University Press.

15. **Young LS**, **Murray PG**. 2003. Epstein-Barr virus and oncogenesis: from latent genes to tumours. Oncogene **22**:5108–21.

16. **Rickinson AB**. 2007. Ovid : Fields Virology Epstein-Barr Virus and Its Replication Ovid : Fields Virology **II**:1–83.

17. **Deyrup AT**. 2008. Epstein-Barr virus-associated epithelial and mesenchymal neoplasms. Hum Pathol **39**:473–83.

18. **Dawson CW**, **Port RJ**, **Young LS**. 2012. The role of the EBV-encoded latent membrane proteins LMP1 and LMP2 in the pathogenesis of nasopharyngeal carcinoma (NPC). Semin Cancer Biol **22**:144–53.

19. **Imai S**, **Sugiura M**. 1994. Gastric carcinoma : Monoclonal epithelial malignant cells expressing Epstein-Barr virus latent infection protein **91**:9131–9135.

20. **Minarovits J**, **Hu LF**, **Imai S**, **Harabuchi Y**, **Kataura A**, **Minarovits-Kormuta S**, **Osato T**, **Klein G**. 1994. Clonality, expression and methylation patterns of the Epstein-Barr virus genomes in lethal midline granulomas classified as peripheral angiocentric T cell lymphomas. J Gen Virol **75 (Pt 1)**:77–84.

21. **Molyneux EM**, **Rochford R**, **Griffin B**, **Newton R**, **Jackson G**, **Menon G**, **Harrison CJ**, **Israels T**, **Bailey S**. 2012. Burkitt's lymphoma. Lancet (London, England) **379**:1234–44.

22. **Burkitt D**. 1962. A children's cancer dependent on climatic factors. Nature **194**:232–4.

23. **Ferry JA**. 2006. Burkitt's lymphoma: clinicopathologic features and differential diagnosis. Oncologist **11**:375–83.

24. **Dalla-Favera R**, **Bregni M**, **Erikson J**, **Patterson D**, **Gallo RC**, **Croce CM**. 1982. Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci U S A **79**:7824–7.

25. **Lenoir GM**, **Preud'homme JL**, **Bernheim A**, **Berger R**. 1982. Correlation between immunoglobulin light chain expression and variant translocation in Burkitt's lymphoma. Nature **298**:474–476.

26. **Taub R**, **Kirsch I**, **Morton C**, **Lenoir G**, **Swan D**, **Tronick S**, **Aaronson S**, **Leder P**. 1982. Translocation of the c-myc gene into the immunoglobulin heavy chain locus in

human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci U S A **79**:7837–41.

27. **Wiman KG**, **Magnusson KP**, **Ramqvist T**, **Klein G**. 1991. Mutant p53 detected in a majority of Burkitt lymphoma cell lines by monoclonal antibody PAb240. Oncogene **6**:1633–9.

28. **Cinti C**, **Claudio PP**, **Howard CM**, **Neri LM**, **Fu Y**, **Leoncini L**, **Tosi GM**, **Maraldi NM**, **Giordano A**. 2000. Genetic alterations disrupting the nuclear localization of the retinoblastoma-related gene RB2/p130 in human tumor cell lines and primary tumors. Cancer Res **60**:383–9.

29. **Cinti C**, **Leoncini L**, **Nyongo A**, **Ferrari F**, **Lazzi S**, **Bellan C**, **Vatti R**, **Zamparelli A**, **Cevenini G**, **Osi GMT**, **Claudio PP**, **Maraldi NM**, **Tosi P**, **Giordano A**. 2000. Genetic Alterations of the Retinoblastoma-Related Gene RB2/p130 Identify Different Pathogenetic Mechanisms in and among Burkitt's Lymphoma Subtypes. Am J Pathol **156**:751–760.

30. **Chappell CP**, **Jacob J**. 2007. Crossroads between Innate and Adaptive Immunity, p. 139–148. *In* Katsikis, PD, Schoenberger, SP, Pulendran, B (eds.), . Springer US, Boston, MA.

31. **Ramiro AR**, **Jankovic M**, **Callen E**, **Difilippantonio S**, **Chen H-T**, **McBride KM**, **Eisenreich TR**, **Chen J**, **Dickins RA**, **Lowe SW**, **Nussenzweig A**, **Nussenzweig MC**. 2006. Role of genomic instability and p53 in AID-induced c-myc-Igh translocations. Nature **440**:105–9.

32. **Ramiro AR**, **Jankovic M**, **Eisenreich T**, **Difilippantonio S**, **Chen-Kiang S**, **Muramatsu M**, **Honjo T**, **Nussenzweig A**, **Nussenzweig MC**. 2004. AID is required for c-myc/IgH chromosome translocations in vivo. Cell **118**:431–8.

33. **Robbiani DF**, **Bothmer A**, **Callen E**, **Reina-San-Martin B**, **Dorsett Y**, **Difilippantonio S**, **Bolland DJ**, **Chen HT**, **Corcoran AE**, **Nussenzweig A**, **Nussenzweig MC**. 2008. AID Is Required for the Chromosomal Breaks in c-myc that Lead to c-myc/IgH Translocations. Cell **135**:1028–1038.

34. **Rowe M**, **Rowel DT**, **Gregory CD**, **Young LS**, **Farrell PJ**, **Rupani H**, **Rickinson AB**. 1987. Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt ' s lymphoma I--cf **6**:2743–2751.

35. **Kamranvar S a**, **Gruhne B**, **Szeles a**, **Masucci MG**. 2007. Epstein-Barr virus

promotes genomic instability in Burkitt's lymphoma. Oncogene **26**:5115–23.

36. **Thorley-Lawson DA**, **Gross A**. 2004. Persistence of the Epstein-Barr virus and the origins of associated lymphomas. N Engl J Med **350**:1328–37.

37. **Paschos K**, **Parker GA**, **Watanatanasup E**, **White RE**, **Allday MJ**. 2012. BIM promoter directly targeted by EBNA3C in polycomb-mediated repression by EBV. Nucleic Acids Res **40**:7233–46.

38. **Vereide DT**, **Seto E**, **Chiu Y-F**, **Hayes M**, **Tagawa T**, **Grundhoff A**, **Hammerschmidt W**, **Sugden B**. 2014. Epstein–Barr virus maintains lymphomas via its miRNAs. Oncogene **33**:1258–1264.

39. **Westhoff Smith D**, **Sugden B**. 2013. Potential cellular functions of Epstein-Barr Nuclear Antigen 1 (EBNA1) of Epstein-Barr Virus. Viruses **5**:226–40.

40. **Xia T**, **O'Hara A**, **Araujo I**, **Barreto J**, **Carvalho E**, **Sapucaia JB**, **Ramos JC**, **Luz E**, **Pedroso C**, **Manrique M**, **Toomey NL**, **Brites C**, **Dittmer DP**, **Harrington WJ**. 2008. EBV MicroRNAs in Primary Lymphomas and Targeting of CXCL-11 by ebv-mir-BHRF1-3. Cancer Res **68**:1436–1442.

41. **Zhou L**, **Bu Y**, **Liang Y**, **Zhang F**, **Zhang H**, **Li S**. 2016. Epstein-Barr Virus (EBV)-BamHI-A Rightward Transcript (BART)-6 and Cellular MicroRNA-142 Synergistically Compromise Immune Defense of Host Cells in EBV-Positive Burkitt Lymphoma. Med Sci Monit **22**:4114–4120.

42. **Harold C**, **Cox D**, **Riley KJ**. 2016. Epstein-Barr viral microRNAs target caspase 3. Virol J **13**:145.

43. **van den Bosch C**. 2004. Is endemic Burkitt's lymphoma an alliance between three infections and a tumour promoter? Lancet Oncol **5**:738–746.

44. **Rochford R**, **Cannon MJ**, **Moormann AM**. 2005. Endemic Burkitt's lymphoma: a polymicrobial disease? Nat Rev Micro **3**:182–187.

45. **Pattle SB**, **Farrell PJ**. 2006. The role of Epstein–Barr virus in cancer. Expert Opin Biol Ther **6**:1193–1205.

46. **Kataaha PK**, **Facert CA**, **Bone EJH**, **Unit J**. 1984. Plasmodium fakiparum products enhance human lymphocyte transformation by Epstein-Barr virus 371–376.

47. **Traggiai E**, **Becker S**, **Subbarao K**, **Kolesnikova L**, **Uematsu Y**, **Gismondo MR**, **Murphy BR**, **Rappuoli R**, **Lanzavecchia A**. 2004. An efficient method to make

human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. Nat Med **10**:871–5.

48. **Zauner L**, **Melroe GT**, **Sigrist J a**, **Rechsteiner MP**, **Dorner M**, **Arnold M**, **Berger C**, **Bernasconi M**, **Schaefer BW**, **Speck RF**, **Nadal D**. 2010. TLR9 triggering in Burkitt's lymphoma cell lines suppresses the EBV BZLF1 transcription via histone modification. Oncogene **29**:4588–98.

49. **Chêne A**, **Donati D**, **Guerreiro-Cacais AO**, **Levitsky V**, **Chen Q**, **Falk KI**, **Orem J**, **Kironde F**, **Wahlgren M**, **Bejarano MT**. 2007. A molecular link between malaria and Epstein-Barr virus reactivation. PLoS Pathog **3**:e80.

50. **Chen F**, **Zou JZ**, **di Renzo L**, **Winberg G**, **Hu LF**, **Klein E**, **Klein G**, **Ernberg I**. 1995. A subpopulation of normal B cells latently infected with Epstein-Barr virus resembles Burkitt lymphoma cells in expressing EBNA-1 but not EBNA-2 or LMP1. J Virol **69**:3752–8.

51. **Tierney RJ**, **Steven N**, **Young LS**, **Rickinson AB**. 1994. Epstein-Barr Virus Latency in Blood Mononuclear Cells : Analysis of Viral Gene Transcription during Primary Infection and in the Carrier State **68**:7374–7385.

52. **Qu L**, **Rowe DT**. 1992. Epstein-Barr Virus Latent Gene Expression in Uncultured Peripheral Blood Lymphocytes **66**:3715–3724.

53. **Babcock BGJ**, **Decker LL**, **Freeman RB**, **Thorley-lawson DA**. 1999. Epstein-Barr Virus – infected Resting Memory B Cells , not Blood of Immunosuppressed Patients **190**.

54. **Joseph a. M**, **Babcock GJ**, **Thorley-Lawson D a.** 2000. EBV Persistence Involves Strict Selection of Latently Infected B Cells. J Immunol **165**:2975–2981.

55. **Babcock GJ**, **Decker LL**, **Volk M**, **Thorley-lawson DA**. 1998. EBV Persistence in Memory B Cells In Vivo **9**:395–404.

56. **Miyashita EM**, **Yang BIN**, **Babcock GJ**, **Thorley-lawson DA**. 1997. Identification of the Site of Epstein-Barr Virus Persistence In Vivo as a Resting B Cell **71**:4882–4891.

57. **Babcock GJ**, **Hochberg D**, **Thorley-lawson DA**. 2000. The Expression Pattern of Epstein-Barr Virus Latent Genes In Vivo Is Dependent upon the Differentiation Stage of the Infected B Cell **13**:497–506.

58. **Thorley-Lawson D a**. 2001. Epstein-Barr virus: exploiting the immune system. Nat

Rev Immunol **1**:75–82.

59. **Price AM**, **Luftig MA**. 2014. Dynamic Epstein–Barr Virus Gene Expression on the Path to B-Cell Transformation, p. 279–313. *In* .

60. **Yao QY**, **Rickinson AB**, **Epstein MA**. 1985. A re-examination of the Epstein-Barr virus carrier state in healthy seropositive individuals. Int J cancer **35**:35–42.

61. **Tan LC**, **Gudgeon N**, **Annels NE**, **Hansasuta P**, **O'Callaghan CA**, **Rowland-Jones S**, **McMichael AJ**, **Rickinson AB**, **Callan MF**. 1999. A re-evaluation of the frequency of CD8+ T cells specific for EBV in healthy virus carriers. J Immunol **162**:1827–35.

62. **Niedobitek G**. 1999. The Epstein-Barr virus : a group 1 carcinogen ? 79–86.

63. **Amon W**, **Farrell PJ**. 2005. Reactivation of Epstein-Barr virus from latency. Rev Med Virol **15**:149–56.

64. **Thorley-Lawson DA**. 2005. EBV the prototypical human tumor virus--just how bad is it? J Allergy Clin Immunol **116**:251–61; quiz 262.

65. **Takada K**. 1984. Cross-linking of cell surface immunoglobulins induces Epstein-Barr virus in Burkitt lymphoma lines. Int J Cancer **33**:27–32.

66. **Baumann M**, **Mischak H**, **Dammeier S**, **Kolch W**, **Gires O**, **Pich D**, **Zeidler R**, **Delecluse HJ**, **Hammerschmidt W**. 1998. Activation of the Epstein-Barr virus transcription factor BZLF1 by 12-O-tetradecanoylphorbol-13-acetate-induced phosphorylation. J Virol **72**:8105–8114.

67. **Countryman J**, **Gradoville L**, **Bhaduri-McIntosh S**, **Ye J**, **Heston L**, **Himmelfarb S**, **Shedd D**, **Miller G**. 2009. Stimulus duration and response time independently influence the kinetics of lytic cycle reactivation of Epstein-Barr virus. J Virol **83**:10694–709.

68. **Packard TA**, **Cambier JC**. 2013. B lymphocyte antigen receptor signaling: initiation, amplification, and regulation. F1000Prime Rep **5**:40.

69. **Cohen J**. 2000. Epstein-Barr Virus Infection. Med Prog 481–492.

70. **Pierce SK**. 2009. Understanding B cell activation: from single molecule tracking, through Tolls, to stalking memory in malaria. Immunol Res **43**:85–97.

71. **Monroe JG**. 2006. ITAM-mediated tonic signalling through pre-BCR and BCR complexes. Nat Rev Immunol **6**:283–294.

72. **Campbell KS**. 1999. Signal transduction from the B cell antigen-receptor. Curr Opin

Immunol **11**:256–64.

73. **Kurosaki T**. 1999. GENETIC ANALYSIS OF B CELL ANTIGEN RECEPTOR SIGNALING. Annu Rev Immunol **17**:555–592.

74. **Seda V**, **Mraz M**. 2015. B-cell receptor signalling and its crosstalk with other pathways in normal and malignant cells. Eur J Haematol **94**:193–205.

75. **Fruehling S**, **Longnecker R**. 1997. The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BCR-mediated signal transduction. Virology **235**:241–251.

76. **Bhende PM**, **Seaman WT**, **Delecluse H-J**, **Kenney SC**. 2004. The EBV lytic switch protein, Z, preferentially binds to and activates the methylated viral genome. Nat Genet **36**:1099–104.

77. **Dickerson SJ**, **Xing Y**, **Robinson AR**, **Seaman WT**, **Gruffat H**, **Kenney SC**. 2009. Methylation-dependent binding of the epstein-barr virus BZLF1 protein to viral promoters. PLoS Pathog **5**:e1000356.

78. **Bergbauer M**, **Kalla M**, **Schmeinck A**, **Göbel C**, **Rothbauer U**, **Eck S**, **Benet-Pagès A**, **Strom TM**, **Hammerschmidt W**. 2010. CpG-methylation regulates a class of Epstein-Barr virus promoters. PLoS Pathog **6**:e1001114.

79. **Countryman J**, **Miller G**. 1985. Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. Proc Natl Acad Sci U S A **82**:4085–9.

80. **Rooney CM**, **Rowe DT**, **Ragot T**, **Farrell PJ**. 1989. The spliced BZLF1 gene of Epstein-Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle. J Virol **63**:3109–16.

81. **Davies AH**, **Grand RJ**, **Evans FJ**, **Rickinson AB**. 1991. Induction of Epstein-Barr virus lytic cycle by tumor-promoting and non-tumor-promoting phorbol esters requires active protein kinase C. J Virol **65**:6838–44.

82. **Goswami R**, **Gershburg S**, **Satorius a**, **Gershburg E**. 2012. Protein kinase inhibitors that inhibit induction of lytic program and replication of Epstein-Barr Virus. Antiviral Res.

83. **Iwakiri D**, **Takada K**. 2004. Phosphatidylinositol 3-kinase is a determinant of responsiveness to B cell antigen receptor-mediated Epstein-Barr virus activation. J

Immunol **172**:1561–6.

84. **Satoh T**, **Hoshikawa Y**, **Satoh Y**, **Kurata T**, **Sairenji T**. 1999. The interaction of mitogen-activated protein kinases to Epstein-Barr virus activation in Akata cells. Virus Genes **18**:57–64.

85. **Binné UK**, **Amon W**, **Farrell PJ**. 2002. Promoter sequences required for reactivation of Epstein-Barr virus from latency. J Virol **76**:10282–9.

86. **Murata T**, **Kondo Y**, **Sugimoto A**, **Kawashima D**, **Saito S**, **Isomura H**, **Kanda T**, **Tsurumi T**. 2012. Epigenetic histone modification of Epstein-Barr virus BZLF1 promoter during latency and reactivation in Raji cells. J Virol **86**:4752–61.

87. **Murata T**, **Narita Y**, **Sugimoto A**, **Kawashima D**, **Kanda T**, **Tsurumi T**. 2013. Contribution of myocyte enhancer factor 2 family transcription factors to BZLF1 expression in Epstein-Barr virus reactivation from latency. J Virol **87**:10148–62.

88. **Ramasubramanyan S**, **Osborn K**, **Flower K**, **Sinclair AJ**. 2012. Dynamic chromatin environment of key lytic cycle regulatory regions of the Epstein-Barr virus genome. J Virol **86**:1809–19.

89. **Yu X**, **Wang Z**, **Mertz JE**. 2007. ZEB1 regulates the latent-lytic switch in infection by Epstein-Barr virus. PLoS Pathog **3**:e194.

90. **Ellis AL**, **Wang Z**, **Yu X**, **Mertz JE**. 2010. Either ZEB1 or ZEB2/SIP1 can play a central role in regulating the Epstein-Barr virus latent-lytic switch in a cell-type-specific manner. J Virol **84**:6139–52.

91. **Kraus RJ**, **Perrigoue JG**, **Mertz JE**. 2003. ZEB negatively regulates the lytic-switch BZLF1 gene promoter of Epstein-Barr virus. J Virol **77**:199–207.

92. **Yu X**, **McCarthy PJ**, **Lim H-J**, **Iempridee T**, **Kraus RJ**, **Gorlen D a**, **Mertz JE**. 2011. The ZIIR element of the Epstein-Barr virus BZLF1 promoter plays a central role in establishment and maintenance of viral latency. J Virol **85**:5081–90.

93. **Geijtenbeek TBH**, **Gringhuis SI**. 2009. Signalling through C-type lectin receptors: shaping immune responses. Nat Rev Immunol **9**:465–79.

94. **Strober W**, **Murray PJ**, **Kitani A**, **Watanabe T**. 2006. Signalling pathways and molecular interactions of NOD1 and NOD2. Nat Rev Immunol **6**:9–20.

95. **Yoneyama M**, **Fujita T**. 2007. Function of RIG-I-like receptors in antiviral innate immunity. J Biol Chem **282**:15315–8.

96. **Kawai T**, **Akira S**. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol **11**:373–84.

97. **Park JS**, **Gamboni-Robertson F**, **He Q**, **Svetkauskaite D**, **Kim J**, **Strassheim D**, **Sohn J**, **Yamada S**, **Maruyama I**, **Banerjee A**, **Ishizaka A**, **Abraham E**. 2006. High mobility group box 1 protein interacts with multiple Toll-like receptors. Am J Physiol Cell Physiol **290**:C917-24.

98. **Vogl T**, **Tenbrock K**, **Ludwig S**, **Leukert N**, **Ehrhardt C**, **van Zoelen M a D**, **Nacken W**, **Foell D**, **van der Poll T**, **Sorg C**, **Roth J**. 2007. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med **13**:1042–9.

99. **Bernard JJ**, **Cowing-Zitron C**, **Nakatsuji T**, **Muehleisen B**, **Muto J**, **Borkowski AW**, **Martinez L**, **Greidinger EL**, **Yu BD**, **Gallo RL**. 2012. Ultraviolet radiation damages self noncoding RNA and is detected by TLR3. Nat Med **18**:1286–90.

100. **Aderem A**, **Ulevitch RJ**. 2000. Toll-like receptors in the induction of the innate immune response. Nature **406**:782–7.

101. **Akira S**, **Takeda K**, **Kaisho T**. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol **2**:675–680.

102. **Slack JL**, **Schooley K**, **Bonnert TP**, **Mitcham JL**, **Qwarnstrom EE**, **Sims JE**, **Dower SK**. 2000. Identification of Two Major Sites in the Type I Interleukin-1 Receptor Cytoplasmic Region Responsible for Coupling to Pro-inflammatory Signaling Pathways * **275**:4670–4678.

103. **Ozinsky A**, **Underhill DM**, **Fontenot JD**, **Hajjar AM**, **Smith KD**, **Wilson CB**, **Schroeder L**, **Aderem A**. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A **97**:13766–71.

104. **Kumar H**, **Kawai T**, **Akira S**. 2009. Toll-like receptors and innate immunity. Biochem Biophys Res Commun **388**:621–5.

105. **Varani S**, **Cederarv M**, **Feld S**, **Tammik C**, **Frascaroli G**, **Landini MP**, **Söderberg-Nauclér C**. 2007. Human cytomegalovirus differentially controls B cell and T cell responses through effects on plasmacytoid dendritic cells. J Immunol **179**:7767–76.

106. **Lund J**, **Sato A**, **Akira S**, **Medzhitov R**, **Iwasaki A**. 2003. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. J Exp

Med **198**:513–20.

107. **Fiola S**, **Gosselin D**, **Takada K**, **Gosselin J**. 2010. TLR9 contributes to the recognition of EBV by primary monocytes and plasmacytoid dendritic cells. J Immunol **185**:3620–31.

108. **Weber F**, **Wagner V**, **Rasmussen SB**, **Hartmann R**, **Paludan SR**. 2006. Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. J Virol **80**:5059–64.

109. **Iwakiri D**, **Zhou L**, **Samanta M**, **Matsumoto M**, **Ebihara T**, **Seya T**, **Imai S**, **Fujieda M**, **Kawa K**, **Takada K**. 2009. Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from Toll-like receptor 3. J Exp Med **206**:2091–9.

110. **Zucchini N**, **Bessou G**, **Traub S**, **Robbins SH**, **Uematsu S**, **Akira S**, **Alexopoulou L**, **Dalod M**. 2008. Cutting edge: Overlapping functions of TLR7 and TLR9 for innate defense against a herpesvirus infection. J Immunol **180**:5799–803.

111. **Månsson A**, **Adner M**, **Höckerfelt U**, **Cardell L-O**. 2006. A distinct Toll-like receptor repertoire in human tonsillar B cells, directly activated by PamCSK, R-837 and CpG-2006 stimulation. Immunology **118**:539–48.

112. **Bourke E**, **Bosisio D**, **Golay J**, **Polentarutti N**, **Mantovani A**. 2003. The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. Blood **102**:956–63.

113. **Bernasconi NL**. 2003. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood **101**:4500–4504.

114. **Hornung V**, **Rothenfusser S**, **Britsch S**, **Krug A**, **Jahrsdörfer B**, **Giese T**, **Endres S**, **Hartmann G**. 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol **168**:4531–7.

115. **Chiron D**, **Bekeredjian-Ding I**, **Pellat-Deceunynck C**, **Bataille R**, **Jego G**. 2008. Toll-like receptors: lessons to learn from normal and malignant human B cells. Blood **112**:2205–2213.

116. **Akira S**. 2004. Toll receptor families: Structure and function. Semin Immunol **16**:1–2.

117. **Medzhitov R**. 2001. Toll-like receptors and innate immunity. Nat Rev Immunol **1**:135–145.

118. **Yamamoto M**, **Takeda K**, **Akira S**. 2004. TIR domain-containing adaptors define the specificity of TLR signaling. Mol Immunol **40**:861–868.

119. **Barton GM**, **Kagan JC**. 2009. A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nat Rev Immunol **9**:535–42.

120. **Häcker BH**, **Vabulas RM**, **Takeuchi O**, **Hoshino K**, **Akira S**, **Wagner H**. 2000. Immune Cell Activation by Bacterial CpG-DNA through Myeloid Differentiation Marker 88 and Tumor Necrosis Factor Receptor – Associated Factor (TRAF) 6. J Exp Med **192**:595–600.

121. **Latz E**, **Schoenemeyer A**, **Visintin A**, **Fitzgerald KA**, **Monks BG**, **Knetter CF**, **Lien E**, **Nilsen NJ**, **Espevik T**, **Golenbock DT**. 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat Immunol **5**:190–8.

122. **Rutz M**, **Metzger J**, **Gellert T**, **Luppa P**, **Lipford GB**, **Wagner H**, **Bauer S**. 2004. Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. Eur J Immunol **34**:2541–2550.

123. **Latz E**, **Verma A**, **Visintin A**, **Gong M**, **Sirois CM**, **Klein DCG**, **Monks BG**, **McKnight CJ**, **Lamphier MS**, **Duprex WP**, **Espevik T**, **Golenbock DT**. 2007. Ligand-induced conformational changes allosterically activate Toll-like receptor 9. Nat Immunol **8**:772–779.

124. **Takeshita F**, **Gursel I**, **Ishii KJ**, **Suzuki K**, **Gursel M**, **Klinman DM**. 2004. Signal transduction pathways mediated by the interaction of CpG DNA with Toll-like receptor 9. Semin Immunol **16**:17–22.

125. **Bekeredjian-Ding I**, **Jego G**. 2009. Toll-like receptors--sentries in the B-cell response. Immunology **128**:311–23.

126. **Akira S**, **Takeda K**. 2004. Toll-like receptor signalling. Nat Rev Immunol **4**:499–511.

127. **Krug A**, **Rothenfusser S**, **Hornung V**, **Jahrsdörfer B**, **Blackwell S**, **Ballas ZK**, **Endres S**, **Krieg AM**, **Hartmann G**. 2001. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. Eur J Immunol **31**:2154–63.

128. **Krieg AM**, **Yi A-K**, **Matson S**, **Waldschmidt TJ**, **Bishop GA**, **Teasdale R**, **Koretzky**

GA, **Klinman DM**. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature **374**:546–549.

129. **Kumagai Y**, **Takeuchi O**, **Akira S**. 2008. TLR9 as a key receptor for the recognition of DNA. Adv Drug Deliv Rev **60**:795–804.

130. **Soni V**, **Cahir-McFarland E**, **Kieff E**. LMP1 TRAFficking Activates Growth and Survival pathways, p. 173–187. *In* TNF Receptor Associated Factors (TRAFs). Springer New York, New York, NY.

131. **Xie P**, **Bishop GA**. 2004. Roles of TNF Receptor-Associated Factor 3 in Signaling to B Lymphocytes by Carboxyl-Terminal Activating Regions 1 and 2 of the EBV-Encoded Oncoprotein Latent Membrane Protein 1. J Immunol **173**:5546–5555.

132. **Martin HJ**, **Lee JM**, **Walls D**, **Hayward SD**. 2007. Manipulation of the toll-like receptor 7 signaling pathway by Epstein-Barr virus. J Virol **81**:9748–58.

133. **Dawson CW**, **George JH**, **Blake SM**, **Longnecker R**, **Young LS**. 2001. The Epstein-Barr virus encoded latent membrane protein 2A augments signaling from latent membrane protein 1. Virology **289**:192–207.

134. **Fukuda M**, **Longnecker R**. 2007. Epstein-Barr virus latent membrane protein 2A mediates transformation through constitutive activation of the Ras/PI3-K/Akt Pathway. J Virol **81**:9299–306.

135. **Wu Y**, **Maruo S**, **Yajima M**, **Kanda T**, **Takada K**. 2007. Epstein-Barr virus (EBV)-encoded RNA 2 (EBER2) but not EBER1 plays a critical role in EBV-induced B-cell growth transformation. J Virol **81**:11236–45.

136. **Nanbo A**, **Takada K**. The role of Epstein-Barr virus-encoded small RNAs (EBERs) in oncogenesis. Rev Med Virol **12**:321–6.

137. **Morrison TE**, **Kenney SC**. 2004. BZLF1, an Epstein-Barr virus immediate-early protein, induces p65 nuclear translocation while inhibiting p65 transcriptional function. Virology **328**:219–32.

138. **Gutsch DE**, **Holley-Guthrie EA**, **Zhang Q**, **Stein B**, **Blanar MA**, **Baldwin AS**, **Kenney SC**. 1994. The bZIP transactivator of Epstein-Barr virus, BZLF1, functionally and physically interacts with the p65 subunit of NF-kappa B. Mol Cell Biol **14**:1939–48.

139. **Ladell K**, **Dorner M**, **Zauner L**, **Berger C**, **Zucol F**, **Bernasconi M**, **Niggli FK**,

Speck RF, **Nadal D**. 2007. Immune activation suppresses initiation of lytic Epstein-Barr virus infection. Cell Microbiol **9**:2055–69.

140. **Brown HJ**, **Song MJ**, **Deng H**, **Wu T**, **Cheng G**, **Sun R**. 2003. NF-kappaB inhibits gammaherpesvirus lytic replication. J Virol **77**:8532–40.

141. **Brumfiel G**. 2008. Language: Disputed definitions. Nature **455**:1023–1028.

142. **Berger SL**, **Kouzarides T**, **Shiekhattar R**, **Shilatifard A**. 2009. An operational definition of epigenetics. Genes Dev **23**:781–783.

143. **Weinhold B**. 2006. Epigenetics: The Science of Change. Environ Health Perspect **114**:160–167.

144. **Jensen P**. 2013. Transgenerational epigenetic effects on animal behaviour. Prog Biophys Mol Biol **113**:447–454.

145. **Goldberg AD**, **Allis CD**, **Bernstein E**. 2007. Epigenetics: a landscape takes shape. Cell **128**:635–8.

146. **Luger K**, **Mäder a W**, **Richmond RK**, **Sargent DF**, **Richmond TJ**. 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature **389**:251–60.

147. **Portela A**, **Esteller M**. 2010. Epigenetic modifications and human disease. Nat Biotechnol **28**:1057–68.

148. **Dawson MA**, **Kouzarides T**. 2012. Cancer epigenetics: from mechanism to therapy. Cell **150**:12–27.

149. **Kim DH**, **Saetrom P**, **Snøve O**, **Rossi JJ**. 2008. MicroRNA-directed transcriptional gene silencing in mammalian cells. Proc Natl Acad Sci U S A **105**:16230–5.

150. **Hurley EA**, **Thorley-Lawson DA**. 1988. B cell activation and the establishment of Epstein-Barr virus latency. J Exp Med **168**:2059–75.

151. **Johannsen E**, **Luftig M**, **Chase MR**, **Weicksel S**, **Cahir-McFarland E**, **Illanes D**, **Sarracino D**, **Kieff E**. 2004. Proteins of purified Epstein-Barr virus. Proc Natl Acad Sci U S A **101**:16286–91.

152. **Kintner C**, **Sugden B**. 1981. Conservation and progressive methylation of Epstein-Barr viral DNA sequences in transformed cells. J Virol **38**:305–16.

153. **Flower K**, **Thomas D**, **Heather J**, **Ramasubramanyan S**, **Jones S**, **Sinclair AJ**. 2011. Epigenetic control of viral life-cycle by a DNA-methylation dependent transcription factor. PLoS One **6**:e25922.

154. **Murata T**, **Noda C**, **Saito S**, **Kawashima D**, **Sugimoto A**, **Isomura H**, **Kanda T**, **Yokoyama KK**, **Tsurumi T**. 2011. Involvement of Jun dimerization protein 2 (JDP2) in the maintenance of Epstein-Barr virus latency. J Biol Chem **286**:22007–16.

155. **Montalvo EA**, **Cottam M**, **Hill S**, **Wang YJ**. 1995. YY1 binds to and regulates cis-acting negative elements in the Epstein-Barr virus BZLF1 promoter. J Virol **69**:4158–65.

156. **Liu P**, **Liu S**, **Speck SH**. 1998. Identification of a negative cis element within the ZII domain of the Epstein-Barr virus lytic switch BZLF1 gene promoter. J Virol **72**:8230–9.

157. **Fernandez AF**, **Rosales C**, **Lopez-Nieva P**, **Grana O**, **Ballestar E**, **Ropero S**, **Espada J**, **Melo SA**, **Lujambio A**, **Fraga MF**, **Pino I**, **Javierre B**, **Carmona FJ**, **Acquadro F**, **Steenbergen RDM**, **Snijders PJF**, **Meijer CJ**, **Pineau P**, **Dejean A**, **Lloveras B**, **Capella G**, **Quer J**, **Buti M**, **Esteban J-I**, **Allende H**, **Rodriguez-Frias F**, **Castellsague X**, **Minarovits J**, **Ponce J**, **Capello D**, **Gaidano G**, **Cigudosa JC**, **Gomez-Lopez G**, **Pisano DG**, **Valencia A**, **Piris MA**, **Bosch FX**, **Cahir-McFarland E**, **Kieff E**, **Esteller M**. 2008. The dynamic DNA methylomes of double-stranded DNA viruses associated with human cancer. Genome Res **19**:438–451.

158. **Woellmer A**, **Arteaga-Salas JM**, **Hammerschmidt W**. 2012. BZLF1 Governs CpG-Methylated Chromatin of Epstein-Barr Virus Reversing Epigenetic Repression. PLoS Pathog **8**.

159. **Miller G**, **El-Guindy A**, **Countryman J**, **Ye J**, **Gradoville L**. 2007. Lytic cycle switches of oncogenic human gammaherpesviruses. Adv Cancer Res **97**:81–109.

160. **Countryman JK**, **Gradoville L**, **Miller G**. 2008. Histone hyperacetylation occurs on promoters of lytic cycle regulatory genes in Epstein-Barr virus-infected cell lines which are refractory to disruption of latency by histone deacetylase inhibitors. J Virol **82**:4706–19.

161. **Zauner L**, **Melroe GT**, **Sigrist J a**, **Rechsteiner MP**, **Dorner M**, **Arnold M**, **Berger C**, **Bernasconi M**, **Schaefer BW**, **Speck RF**, **Nadal D**. 2010. TLR9 triggering in Burkitt's lymphoma cell lines suppresses the EBV BZLF1 transcription via histone modification. Oncogene **29**:4588–4598.

162. **Kondo Y**. 2009. Epigenetic cross-talk between DNA methylation and histone modifications in human cancers. Yonsei Med J **50**:455–63.

163. **Saccani S**, **Natoli G**. 2002. Dynamic changes in histone H3 Lys 9 methylation occurring at tightly regulated inducible inflammatory genes. Genes Dev **16**:2219–24.

164. **Foster SL**, **Hargreaves DC**, **Medzhitov R**. 2007. Gene-specific control of inflammation by TLR-induced chromatin modifications. Nature **447**:972–8.

165. **Albrecht I**, **Tapmeier T**, **Zimmermann S**, **Frey M**, **Heeg K**, **Dalpke A**. 2004. Toll-like receptors differentially induce nucleosome remodelling at the IL-12p40 promoter. EMBO Rep **5**:172–7.

166. **Weinmann AS**, **Mitchell DM**, **Sanjabi S**, **Bradley MN**, **Hoffmann A**, **Liou H-C**, **Smale ST**. 2001. No Title. Nat Immunol **2**:51–57.

167. **Vermeulen L**, **Vanden Berghe W**, **Beck IME**, **De Bosscher K**, **Haegeman G**. 2009. The versatile role of MSKs in transcriptional regulation. Trends Biochem Sci **34**:311–8.

168. **Ananieva O**, **Darragh J**, **Johansen C**, **Carr JM**, **McIlrath J**, **Park JM**, **Wingate A**, **Monk CE**, **Toth R**, **Santos SG**, **Iversen L**, **Arthur JSC**. 2008. The kinases MSK1 and MSK2 act as negative regulators of Toll-like receptor signaling. Nat Immunol **9**:1028–1036.

169. **Picard C**, **Puel A**, **Bonnet M**, **Ku C**, **Bustamante J**, **Yang K**, **Soudais C**, **Dupuis S**, **Feinberg J**, **Fieschi C**, **Elbim C**, **Hitchcock R**, **Lammas D**, **Davies G**, **Al-Ghonaium A**, **Al-Rayes H**, **Al-Jumaah S**, **Al-Hajjar S**, **Al-Mohsen IZ**, **Frayha HH**, **Rucker R**, **Hawn TR**, **Aderem A**, **Tufenkeji H**, **Haraguchi S**, **Day NK**, **Good RA**, **Gougerot-Pocidalo M-A**, **Ozinsky A**, **Casanova J-L**. 2003. Pyogenic bacterial infections in humans with IRAK-4 deficiency. Science **299**:2076–9.

170. **Lye E**, **Mirtsos C**, **Suzuki N**, **Suzuki S**, **Yeh WC**. 2004. The role of interleukin 1 receptor-associated kinase-4 (IRAK-4) kinase activity in IRAK-4-mediated signaling. J Biol Chem **279**:40653–40658.

171. **Motshwene PG**, **Moncrieffe MC**, **Grossmann JG**, **Kao C**, **Ayaluru M**, **Sandercock AM**, **Robinson C V**, **Latz E**, **Gay NJ**. 2009. An oligomeric signaling platform formed by the Toll-like receptor signal transducers MyD88 and IRAK-4. J Biol Chem **284**:25404–11.

172. **Hagemeier SR**, **Dickerson SJ**, **Meng Q**, **Yu X**, **Mertz JE**, **Kenney SC**. 2010. Sumoylation of the Epstein-Barr virus BZLF1 protein inhibits its transcriptional activity and is regulated by the virus-encoded protein kinase. J Virol **84**:4383–94.

173. **Duellman SJ**, **Thompson KL**, **Coon JJ**, **Burgess RR**. 2009. Phosphorylation sites of Epstein-Barr virus EBNA1 regulate its function. J Gen Virol **90**:2251–2259.

174. **Flemington E**, **Speck SH**. 1990. Autoregulation of Epstein-Barr virus putative lytic switch gene BZLF1. J Virol **64**:1227–32.

175. **Speck SH**, **Chatila T**, **Flemington E**. 1997. Reactivation of Epstein-Barr virus: regulation and function of the BZLF1 gene. Trends Microbiol **5**:399–405.

176. **Montalvo EA**, **Shi Y**, **Shenk TE**, **Levine AJ**. 1991. Negative regulation of the BZLF1 promoter of Epstein-Barr virus. J Virol **65**:3647–55.

ATTACHMENTS

Manuscript I:

IRAK4 is essential for TLR9-induced suppression of Epstein-Barr virus *BZLF1* transcription in Akata Burkitt's lymphoma cells

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Abstract

Latent Epstein-Barr virus (EBV) infection is associated with endemic Burkitt's lymphoma (eBL) that is epidemiologically linked to chronic infection with the malaria agent *Plasmodium falciparum*. Lytic reactivation of EBV eventually leads to the death of the host cell. Previously, we showed that activation of the innate immunity signaling via Toll-like receptor (TLR) 9 by hemozoin, a pigment of *P. falciparum,* is critical in inhibiting EBV lytic reactivation and thereby causing reinforcement of its latency in eBL cells. Here, we show that EBV lytic repression is reinforced by TLR9 engagement via a mechanism that is largely protein synthesis independent and that two TLR9 downstream signaling molecules, MyD88 and IRAK4, exhibit central roles. Counteracting signaling of these molecules could allow lytic reactivation and thus provide novel treatment targets to combat eBL.

Keywords: TLR9; Burkitt's lymphoma; Epstein–Barr virus; *Plasmodium falciparum*;

Importance

Latent Epstein-Barr virus (EBV) infection is associated with endemic Burkitt's lymphoma (eBL) that is epidemiologically linked to chronic infection with the malaria agent Plasmodium falciparum. Lytic reactivation of EBV eventually leads to the death of the host cell. Previously, we showed that activation of the innate immunity signaling via Toll-like receptor (TLR) 9 by hemozoin, a pigment of *P. falciparum*, is critical in inhibiting EBV lytic reactivation and thereby causing reinforcement of its latency in eBL cells. Here, we show that EBV lytic repression is reinforced by TLR9 engagement via a mechanism that is largely protein synthesis independent and that two TLR9 downstream signaling molecules, MyD88 and IRAK4, exhibit central roles. Counteracting signaling of these molecules could allow lytic reactivation and thus provide novel treatment targets to combat eBL.

Introduction

Primary infection with the Epstein-Barr virus (EBV) is mostly asymptomatic, and more than 90% of the adult population worldwide are EBV carriers after the virus has established reversible latent infection [1,2]. This life-long, virtually harmless, host–virus coexistence must be regarded as the result of a long co-evolution based on modulation of EBV gene expression in different subsets of infected cells and the finely tuned adaptation to the immune response of the human host [3]. Yet, EBV is associated with endemic Burkitt's lymphoma (eBL), one of the most common cancers in children in equatorial Africa, i.e., in areas where chronic co-infection with EBV and the malaria parasite *Plasmodium falciparum* prevails [4]. As a member of the gammaherpesvirus family, EBV establishes latency in B cells [5]. In eBL cells, EBV persists in a highly restricted form of latency [6], termed latency program I. In this program, EBV's lytic and latent genes are repressed except EBV nuclear antigen (EBNA)1, which is essential for episomal retention of EBV in dividing cells. Thereby, the propagation of the virus to daughter cells is guaranteed and the repression of EBV's gene expression contributes to the evasion from the host's immune system [7].

Latency of EBV is reversible, to ensure viral transmission to uninfected cells and to new hosts [2]. Thus, EBV periodically lytically reactivates, which leads to the production of infectious viral particles and death of the infected B-cell. The lytic reactivation is set off by the expression of the immediate-early protein ZEBRA encoded by EBV's master lytic gene *BZLF1*. ZEBRA is a transcription factor that induces a lytic cascade leading to early and late lytic EBV gene expression [8]. In BL cell lines, latently infected with EBV (e.g. Akata cells), the lytic reactivation can be induced using diverse agents including phorbol esters (TPA), sodium butyrate (SB), transforming growth factor-β (TGF-β), and B-cell

receptor (BCR) cross-linking anti-immunoglobulin G (anti-IgG) [9–13]. EBV particles and lytic proteins provoke a wide range of immune responses through innate immune mechanisms [14] and adaptive humoral [15] and T-cell responses [16]. Thus, the restriction to essential proteins required for the latent viral persistence and replication is beneficial for both EBV and the host cell. To prevent aberrant spontaneous lytic reactivation, cell death and subsequent activation of the immune system, EBV latency is tightly controlled by histone modifications [17,18] and by DNA methylation [19]. In addition to these intrinsic regulating factors, EBV can hijack the innate immune system, and, in particular, the signaling via Toll-like receptors (TLRs) to regulate the balance between latency and lytic reactivation [20]. Our group showed that hemozoin, a disposal product formed from the digestion of blood by *P. falciparum*, activates TLR9 signaling and suppresses the lytic reactivation of EBV in BL B-cells *in vitro* by affecting the histones state at the promoter of *BZLF1* [21]. Nevertheless, it remains unclear whether this effect is protein synthesis independent, thus a direct consequence of the activation of TLR9 signaling, or whether it requires the expression of a specific unknown protein.

TLRs are essential elements of the innate immune system. They are transmembrane receptors involved in the recognition of pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs), which initiate the inflammatory response by the production of cytokines [22,23]. Endosomal TLR9 is expressed in B cells and acts as a sensor for unmethylated CpG oligonucleotides (ODN) found on a large scale in bacterial DNA [24]. Upon stimulation, the TLR9 cytoplasmic Toll/interleukin-1 receptor (TIR) domain associates with the TIR domain-containing adaptor myeloid differentiation primary response gene 88 (MyD88). The latter recruits the interleukin-1 receptor-associated kinase (IRAK) 4 to TLR9 through interaction of the death domains of both molecules. IRAK-1 is activated by phosphorylation and associates

with the TNF receptor associated factor (TRAF) 6, thereby activating the IκB kinase (IKK) complex, leading to activation of mitogen-activated protein (MAP) kinases (JNK, p38, MAPK) and of nuclear factor kappa B (NF-κB). NF-κB promotes the transcription of genes involved in the cellular activation, proliferation and in the production of pro-inflammatory cytokines [25]. Recently, we showed that several elements of the TLR9 signaling pathway, including NF-κB, PI3K, ERK, JNK and p38, are not necessary for the inhibitory effect of TLR9 signaling on *BZLF1* mRNA expression [21]. Thus, additional investigation is required to precisely delineate how TLR9 signaling impacts on EBV lytic reactivation. Understanding the mechanisms favoring maintenance of lytic EBV infection could provide potential targets for treatments aiming at activating lytic EBV replication and inducing lysis of EBV-harboring cancer B cells.

Here, we aimed at advancing the detailed understanding of TLR9 stimulation suppressing EBV lytic reactivation. Using protein synthesis inhibitors, we investigated whether inhibition of EBV lytic reactivation requires *de novo* protein expression, or if it runs through already existing elements. Moreover, we tested the importance of key components of the signaling pathway, which are directly downstream of TLR9 by generating Akata BL cells with either silenced or inactivated *TLR9*, *MyD88*, *IRAK4,* or *IRAK1* genes.

Material and Methods

Cells and cell culture

The BL cell line Akata [12] was obtained from Dr. Andrew Bell (Birmingham, UK). Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, streptomycin (100 mg/ml), penicillin (100 U/ml) and L-glutamine (2 mM). Akata cells transfected with the shLucGL3 (control) or shIRAK4 expressing plasmids were grown in complete medium supplemented with 200 µg/ml Zeocin (InvivoGen, Nunningen, Switzerland). Akata cells expressing a dominant-negative MyD88 (DN-MyD88 Akata) [21] were grown in the same medium supplemented with 0.4 mg/ml G418 (Promega, Dübendorf, Switzerland).

Reagents and antibodies

ODN 2006 type B CpG oligonucleotide (# 11B15-MM) was bought from InvivoGen. The polyclonal rabbit α-human-IgG antibody (#A0423) was obtained from Dako (DakoCytomation, Zug, Switzerland). Cycloheximide (VWR international, Dietikon, Switzerland) was dissolved in H_2O to a concentration of 10 mg/ml, whereas 4E1RCat (Sigma-Aldrich, Buchs, Switzerland) was adjusted to a concentration of 500 µg/ml in DMSO. Digitonin was dissolved in DMSO to a concentration of 20 mg/ml.

EBV lytic reactivation and ODN CpG 2006 stimulation

Akata cells were resuspended at 1 x 10^6 cells/ml in supplemented RPMI 1640 and stimulated with 0.5 mM end concentration ODN CpG 2006 (InvivoGen) 2 h prior to stimulation with 100 µg/ml anti-IgG (Dako). At 6 h after treatment with anti-IgG, cell pellets were harvested for RNA and protein extraction.

Protein synthesis inhibition

For protein synthesis inhibition, Akata cells were treated with 4E1RCat (10 μM or 25 μM) or cycloheximide (33 μg/ml) for 30 min before ODN CpG 2006 treatment. Cells were then treated as described in the section above. The cells viability was assed by Trypan Blue exclusion assay.

hIL-10 ELISA

hIL-10 protein concentrations were determined in supernatants from stimulated cultures by standard capture ELISA (Ready- SET-Go, eBioscience, Vienna, Austria) according to the manufacturer's instructions. Plates were read using a Synergy HT Multi-Detection Microplate Reader (BioTek, Luzern, Switzerland) at 450 nm and 570 nm. The values measured at 570 nm were subtracted from those of 450 nm and the cytokine concentration was determined by extrapolation from the standard curve.

RNA preparation, reverse transcription and RT-qPCR (TaqMan)

RNA isolation, DNAse treatment, reverse transcription and quantitative PCR (RT-qPCR) was performed as described before [20,26]. *BZLF1*, *hIL-10*, *C-myc* (Hs00153408_m1; Life Technologies, Zug, Switzerland), *IRAK4* (Hs00211616_m1; Life Technologies) or *TLR9* (Hs00152973_m1; Life Technologies) mRNA expression was normalized to the mRNA of the housekeeping gene *HMBS* (same as above) resulting in Δ cycle threshold (ΔCT) values.

Cell lysis and Western blot

Total protein lysates were obtained after lysing 10^6 cells in RIPA complete buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 2mM EDTA, 1% NP40 complemented with 0.1% SDS, 1 x EDTA-free protease inhibitor cocktail (Roche, Rotkreuz, Switzerland)). Cell extracts were passed 10 times through a 25-G syringe. Protein content was determined using the Pierce BCA Protein Assay Kit (ThermoScientific, Zug, Switzerland), according to the manufacturer's instructions. To analyze protein expression by western blot, protein (20 mg/well) was loaded into a NuPAGE 4–12% Bis-Tris Gel (Life Technologies), subjected to SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare, Glattbrugg, Switzerland). The membrane was incubated with rabbit anti-IRAK1 (sc-7883, Santa Cruz Biotech, Santa Cruz, CA), rabbit anti-Akt (#9272), rabbit anti-pAkt (#4058), rabbit anti-Mek1/2 (#9122), rabbit anti-pMek1/2 (#2338), rabbit anti-Syk (#12358), rabbit anti-pSyk (#2710) or rabbit anti-β-actin (# 4967, all from Cell Signaling Technology, Allschwil, Switzerland) primary antibodies; subsequently with anti-rabbit (# 7074) or anti-mouse (# 7076) IgG HRP-linked secondary antibodies (Cell Signaling Technology). The signal was detected with the ECL Western Blotting Detection Reagents (GE Healthcare) and imaged using the LAS-3000 image reader (Fujifilm, Dielsdorf, Switzerland).

EBV genome measurement

Genomic DNA was extracted from 2 x 10^6 Akata cells using the QIAamp DNA Mini Kit (Qiagen, Hombrechtikon, Switzerland). Primers were diluted to a concentration of 300 nM with 150 ng DNA, 1x SYBR green MasterMix (#4309155, Life Technologies) and filled with water to a volume of 10 µl. Samples were measured in triplicates. Measurements were carried out on an ABI 7900HT Fast real-time PCR system (Applied

Biosystems, Rotkreuz, Switzerland) and analyzed with SDS 2.2 software. SYBR green primers were as following:

genomic BamH1 W: forward: GCCAGACAGCAGCCAATTGT; reverse: GACTCCTGGCGCTCTGATG*; genomic HMBS*: forward: ACCAGCTCCCTGCGAAGAG; reverse: GAACTCCAGATGCGGGAACTT

CRISPR/Cas9 genome editing

Single guide RNA sequences targeting IRAK4, IRAK1, MyD88 and TLR9 were designed using the CRIPR Design webtool (http://crispr.mit.edu/). The three sequences having the lowest number of off-target sites were selected for each gene (**Table 1, Figure 1**). Complementary oligonucleotides containing the guide RNA (without PAM sequence) and BpiI ligation adapter were synthesized by Microsynth (Balgach, Switzerland). The annealed oligos were ligated into BpiI digested pSpCas9(BB)-2A-GFP (px458) vector, a gift from Feng Zhang (Addgene plasmid # 48138). The sequence of the constructs was verified by DNA sequencing. CRISPR single cell clones were obtained by electroporation (Neon® Transfection system, ThermoFisher Scientific, Zug, Switzerland) of Akata cells with the px458 plasmids. Transfected single cells, positive for green fluorescent protein (GFP), were sorted 48h after transfection using a FACS ARIA II cell sorter (BD Biosciences, Allschwil, Switzerland). Single cell clones were genotyped and characterized after expansion (**Table 2, Figure 1**).

Statistics

Level of significance was evaluated by an unpaired Student's *t* test or Tukey's multiple comparison test (ANOVA) using Prism 6 (GraphPad Software, Inc.). P levels < 0.05 were regarded as statistically significant.

Results

TLR9-induced inhibition of lytic BZLF1 expression is largely protein synthesis independent

Hemozoin, a pigment expressed by the malaria agent *Plasmodium falciparum*, activates the TLR9 pathway [21,27]. We have previously shown that TLR9-triggering by ODN CpG 2006 or Hemozoin inhibits EBV lytic reactivation, i.e. expression of *BZLF1*, induced by BCR cross-linking in BL cells *in vitro* [21]. Here, we aimed to elucidate the mechanism by which TLR9 inhibits *BZLF1* expression. In particular, we wanted to understand if *BZLF1* expression is inhibited directly e.g. through post-translational modification of existing proteins induced by the TLR9 signaling, or indirectly, through *de novo* expression of proteins, e.g. of a transcription or repressor factor expressed upon TLR9 stimulation.

We treated Akata BL cells with a protein synthesis inhibitor, either cycloheximide or 4E1RCat. We chose these inhibitors because they act at the translational level and should not influence *BZLF1* mRNA levels. First, in order to determine the cytotoxicity of the protein synthesis inhibitors, we assessed the cell viability 8.5h after treatment of Akata BL cells with 33 µg/ml of cycloheximide, or 10 µM and 25 µM of 4E1RCat, respectively (**Fig. 2a**). Treatment with 33 µg/ml cycloheximide, or 10 µM 4E1RCat did not affect cell viability, whereas 25 µM 4E1RCat or vehicle treatment, with the same volume DMSO as for 25 µM 4E1RCat, decreased cell viability compared to untreated cells to 60% (p=0.554), and to 73% (p=0.23), respectively. The treatment of the cells with digitonin, used as positive control, significantly reduced the cell viability to 30% (p=0.0034) (**Fig. 2a**)..

Akata BL cells first treated with protein synthesis inhibitors, cycloheximide or 4E1RCat for 30 min, were tested for EBV lytic reactivation after additional treatment

with ODN CpG 2006 or/and anti-IgG. After 6h treatment with anti-IgG, EBV lytic reactivation was assessed by the measurement of the increase of the mRNA expression of the immediate early lytic transcription factor *BZLF1* (**Fig. 2b and c**). Treatment with ODN CpG 2006, cycloheximide or 4E1RCat had *per se* no effect on *BZLF1* mRNA expression levels (**Fig. 2b**). In cells with no protein synthesis inhibition, activation of TLR9 with ODN CpG 2006 inhibited the *BZLF1* mRNA increase after BCR cross-linking with anti-IgG, and thus lytic reactivation, by 83.7% (p=0.0317) (**Fig. 2c**). Treatment with cycloheximide or 4E1RCat led to respectively a 14-times and to an 11-times lower *BZLF1* mRNA expression after BCR triggering via anti-IgG compared to cells with no protein synthesis inhibition. This was expected, since expression of *BZLF1* upon BCR crosslinking is reinforced by a positive feedback loop exerted by ZEBRA, the protein expressed by the *BZLF1* gene, on its own promoter [28–30] (**Fig. 2c**). Importantly, upon protein synthesis inhibition with cycloheximide or 10 μM 4E1RCat, TLR9 triggering via ODN CpG 2006 inhibited *BZLF1* mRNA increase by 77.7% (p=0.0418) and by 78.7 % (p=0.011), respectively. Therefore, TLR9 stimulation can inhibit *BZLF1* expression directly through a largely protein synthesis independent mechanism.

TLR9-induced inhibition of EBV lytic reactivation is MyD88, IRAK4 and IRAK1 dependent in Burkitt's lymphoma cells

As shown above, TLR9 activation triggered inhibition of EBV lytic reactivation is largely *de novo* protein synthesis independent and is strictly MyD88-dependent [21]. However, the downstream TLR9 signaling elements, NF-κB, phosphatidylinositol-3 kinase (PI3K), extra-cellular signal regulated kinase (ERK), *c-jun* N terminal kinase (JNK) and p38, are not fully responsible for TLR9-dependent *BZLF1* inhibition [21]. IRAK4 and IRAK1

molecules, which act immediately downstream of MyD88, were not tested. Thus, to know the importance of IRAK4 and IRAK1 proteins in TLR9-induced inhibition of *BZLF1* expression, we used the CRISPR/Cas9 genome editing method [31] to inactivate IRAK4 and IRAK1, respectively. In separate experiments, TLR9 and MyD88 were also inactivated to further confirm our previous results, and to use the TLR9 or MyD88 inactivated cells as positive controls.

After transfection of Akata cells with modified Cas9-gRNA-GFP (px458) expression plasmids (**Fig. 1**), GFP-positive single cells were sorted and amplified. The clones were sequenced and 2-3 clones containing an early stop codon in the TLR9, MyD88, IRAK4, or IRAK1 coding sequences were selected for further characterization (**Table 2**). To verify that every cell clone had the same receptors expression level, IgG protein expression on the cells' surface was characterized by flow cytometry (**Fig. 3a**), and for TLR9 mRNA levels by were measured by RT-qPCR (**Fig. 3b**). We then measured IL-10 protein levels in the supernatants (**Fig. 3c**) and the NF-κB nuclear translocation (**Fig. 4**), after 8h treatment with ODN CpG 2006, as readouts for the integrity of the TLR9 pathway activity. Finally, we amplified the conserved EBV *BamHI W* sequence, present in multiple copies on the viral genome, by RT-qPCR to determine the EBV presence in the cell clones.

None of the inactivating mutations did affect the BCR surface expression levels of the cloned cells as determined by flow cytometry. 81.1% of the Akata WT cells expressed IgG on the surface, whereas 90.4 % of the IRAK4$^-$ a3, 94.3 % of the IRAK4$^-$ a4, 92.3 % of the IRAK1$^-$ b2, 93.1 % of the IRAK1$^-$ b8, 91.8 % of the TLR9$^-$ b3, 91.7 % of the TLR9$^-$ b5, 92.7 % of the TLR9$^-$ b6, 92.9 % of the MyD88$^-$ a3, 94.2 % of the MyD88$^-$ a5 and 95.3 % of the MyD88$^-$ b1 cell clones were IgG positive (**Fig. 3a**). The *TLR9* mRNA levels in the clones ranged between 0.5-fold and 1.2-fold of the mRNA levels measured in the

Akata WT cells (**Fig. 3b**). On the other hand, the 5.5-fold IL-10 protein level increase measured after 8h ODN CpG 2006 treatment of WT Akata cells was not observed in the IRAK4$^-$ a3, IRAK4$^-$ a4, IRAK1$^-$ b2, IRAK1$^-$ b8, TLR9$^-$ b3, TLR9$^-$ b5, TLR9$^-$ b6, MyD88$^-$ a3, MyD88$^-$ a5 and MyD88$^-$ b1 cell clones, (**Fig. 3c**) indicating that the inactivation of the TLR9 pathway was not a consequence of the variation in TLR9 mRNA levels. We confirmed the inactivation of the TLR9 pathway by measuring the NF-κB nuclear translocation by WB. After 90 min treatment with ODN CpG 2006, Akata WT cells showed a strong nuclear NF-κB protein levels increase compared to untreated cells, on the other hand in IRAK4$^-$ a3, IRAK4$^-$ a4, TLR9$^-$ b3, TLR9$^-$ b5, TLR9$^-$ b6, MyD88$^-$ a3, MyD88$^-$ a5 and MyD88$^-$ b1 cell clones the nuclear NF-κB was not increased by ODN CpG 2006 treatment (**Fig. 4**). The selected cell clones IRAK4$^-$a3, IRAK4$^-$a4, TLR9$^-$b3, TLR9$^-$b5, TLR9$^-$b6, MyD88$^-$a3, MyD88$^-$a5 and MyD88$^-$b1 contained between 20 and 95 *BamHI W* copies per cell. For comparison the Akata WT cells contained about 85 *BamHI W* copies (**Fig. 3d**).

Despite variable EBV DNA copy numbers per cell, the detection of *BamHI W* copies allowed us to use the cell clones IRAK4$^-$ a3, IRAK4$^-$ a4, TLR9$^-$ b3, TLR9$^-$ b5, TLR9$^-$ b6, MyD88$^-$ a3, MyD88$^-$ a5 and MyD88$^-$ b1 for further *BZLF1* expression assays. By contrast and surprisingly, the *BamHI W* sequence was not detectable in IRAK1 cell clones IRAK1$^-$ b2 and IRAK1$^-$ b8, kept about 1 month in culture for the amplification step after single cell cloning. For this reason, the *BZLF1* reactivation upon BCR cross-linking was not detectable anymore in the IRAK1$^-$ clones. The transfection, selection and amplification steps were therefore repeated using IRAK1$^-$b px458 plasmid. In order to control that the loss of EBV copies in IRAK1 inactive clones was not due to the long culture time required for the amplification step, Akata cells transfected with a control px458 plasmid lacking a sgRNA target sequence, as well as untransfected Akata WT cells

were isolated and amplified in parallel. The cells were directly tested for the TLR9 pathway activity by the measurement of IL-10 mRNA and proteins in the supernatant, as well as for the presence of EBV by the measurement of *BamHI W* copies (**Table 3**). To sum up the CRISPR/Cas9 plasmid transfection experiments, Akata cells transfected with control px458 plasmid as well as untransfected Akata single cell clones all had a active TLR9 pathway and about 83.3% of the clones where EBV positive. This shows that the control px458 plasmid and the transfection, selection and amplification steps necessary to obtain single cell clones had no influence on the TLR9 pathway activity and the EBV presence in the cells. On the other hand, respectively 56.5%, 40%, 50% and 60% of the IRAK1$^-$, IRAK4$^-$, TLR9$^-$ and MyD88$^-$ px458 transfected clones had an inactive TLR9 pathway. Interestingly, respectively 75%, 60% and 83.3% of the IRAK4$^-$, TLR9$^-$ and MyD88$^-$ px458 transfected clones that had an inactive TLR9 pathway and were EBV positive (**Table 3**). In IRAK1$^-$b px458 transfected cell clones, about 56.5 % of the clones had an inactive TLR9 pathway, and all had completely lost EBV genome (**Table 3**), which could suggests a role for IRAK1 in EBV viral persistence and transmission to dividing daughter cells. For this reason, the *BZLF1* reactivation upon BCR cross-linking was not detectable anymore in the IRAK1$^-$ clones.

Next, in order to determine the importance of IRAK4 in the TLR9-induced inhibition of *BZLF1* expression the CRISPR/Cas9 mutated clones were treated with ODN CpG 2006 for 2h, followed by treatment with anti-IgG for 6h. TLR9 and MyD88 knockouts were used as controls. EBV lytic reactivation was determined by the measurement of *BZLF1* mRNA levels by RT-qPCR. For the IRAK4 inactive clones IRAK4$^-$ a3 and IRAK4$^-$ a4, we measured 194 and 463 *BZLF1* relative transcripts over *HMBS* over untreated sample after BCR cross-linking, respectively (**Fig. 5a**); and for the TLR9 inactive clones TLR9$^-$ b3, TLR9$^-$ b5 and TLR9$^-$ b6, we measured 789, 627 and 940 *BZLF1*

relative transcripts over *HMBS* over untreated sample after BCR cross-linking, respectively (**Fig. 5b**). For comparison, for WT Akata cells we measured 1583 *BZLF1* relative transcripts over *HMBS* over untreated sample after BCR cross-linking. In the MyD88 inactive clones MyD88$^-$ a3, MyD88$^-$ a5 and MyD88$^-$ b1 we measured 1623.8, 1632 and 1837 *BZLF1* relative transcripts over *HMBS* over untreated sample after BCR cross-linking, respectively (**Fig. 5c**).

The basal *BZLF1* mRNA levels measurement in untreated and ODN CpG 2006 treated cells shows that the differences of spontaneous lytic reactivation between the TLR9-, MyD88- and IRAK4- CRISPR/Cas9 clones compared to WT Akata cells, are minor, although statistically significant for TLR9-b5, MyD88-a3 and MyD88-b1 clones (**Supplementary Fig. S1**). Moreover, the differences in basal *BZLF1* mRNA levels do not correlate with the EBV DNA copies measured in each clones (**Fig. 3d**) and neither with the EBV lytic reactivation upon BCR cross-linking (**Fig. 5**). Spontaneous lytic reactivation is therefore, not the reason explaining neither the differences in EBV DNA loads nor the differences in *BZLF1* mRNA expression upon BCR cross-linking.

In order to investigate if the BCR signaling is affected by the mutation we stimulated the IRAK4$^-$ a3, IRAK4$^-$ a4, TLR9$^-$ b3, TLR9$^-$ b5 and TLR9$^-$ b6 clones with anti-IgG for 15, 30, 60 and 120 minutes. The phosphorylation levels of Akt, Mek and Syk were measured in stimulated CRISPR/Cas9 clones by western blot and compared with the levels in Akata WT cells. The results show an increased Akt, Mek and Syk phosphorylation upon treatment with anti-IgG with only minor differences between the IRAK4$^-$ and TLR9$^-$ clones and the Akata WT cells (**Supplementary Fig. S2, S3 and S4**). From these results we exclude an influence of IRAK4 or TLR9 abrogation on the BCR pathway to be the reason for the differences in *BZLF1* mRNA expression upon lytic reactivation.

The activation of TLR9 by ODN CpG 2006 in WT Akata cells reduced the expression of *BZLF1* mRNA about 4.5 times to 379.7 (p=0.0005) relative transcripts after BCR cross-linking. Interestingly, we showed that, despite differences in the *BZLF1* mRNA level after BCR cross-linking between the clones and WT Akata cells, the TLR9-induced reduction of *BZLF1* mRNA expression was completely rescued in the IRAK4$^-$a3 and IRAK4$^-$a4 clones with 264.6 (p=0.0363) respectively 527.3 (p=0.1855) *BZLF1* relative transcripts (**Fig. 5a**); and in the TLR9$^-$b3, TLR9$^-$b5 and TLR9$^-$b6 control clones, with respectively 855.5 (p=0.0112), 527.2 (p=0.078) and 998.1 (p=0.2435) *BZLF1* relative transcripts (**Fig. 5b**); and in the MyD88$^-$ a3, MyD88$^-$ a5 and MyD88$^-$ b1 control clones with respectively 1408.6 (p=0.2998), 1861.2 (p=0.3735) and 1682.9 (p=0.064) *BZLF1* relative transcripts (**Fig. 5c**). To sum up, by transfecting Akata cells with CRISPR/Cas9 plasmids containing sgRNA targeting IRAK4, TLR9 or MyD88 genes, insertions or deletions could be induced leading to an early stop codon in the target genes. The characterization of the single cell clones showed that the surface IgG protein level remained stable and comparable to the levels measured in WT Akata cells; that the TLR9 pathway activity was completely abrogated by the early stop codon in the target genes; and not as a consequence of the variation in TLR9 mRNA level; finally, despite variable EBV DNA copy numbers per cell, the IRAK4$^-$ a3, IRAK4$^-$ a4, TLR9$^-$ b3, TLR9$^-$ b5, TLR9$^-$ b6, MyD88$^-$ a3, MyD88$^-$ a5 and MyD88$^-$ b1 clones could be used for lytic reactivation assays.

In conclusion, these results show that in BL cells the TLR9-induced inhibition of lytic reactivation requires functional IRAK4, TLR9 and MyD88 proteins.

Discussion

EBV lytic reactivation upon BCR cross-linking is inhibited by TLR9 stimulation with ODN CpG 2006 in B cells [21]. Here, we studied the importance of protein synthesis, and the involvement of the signaling proteins MyD88, IRAK4 and IRAK1, in this process. We found that (i) TLR9-induced inhibition of EBV lytic reactivation in B-cells upon BCR cross-linking, is largely independent from de novo protein synthesis; (ii) the inactivation of either TLR9, MyD88, or IRAK4 completely abrogates the effect of ODN CpG 2006 on TLR9 signaling resulting in unhindered EBV lytic reactivation upon BCR cross-linking in the presence of ODN CpG 2006. Our results unprecedentedly show that TLR9 triggering activates a signaling pathway that does not only depend on protein synthesis to favor EBV latency in B cells upon stimulation of the BCR; and demonstrate the central roles of MyD88 and IRAK4 in this mechanism contributing to EBV's persistence in the host's B-cell pool.

Remarkably, activation of TLR9 by ODN CpG 2006 inhibited the transcription of EBV's master lytic gene *BZLF1* in BL cells to a similar degree, even when mRNA translation was strongly inhibited. To direct our investigation, we tackled the question whether protein synthesis is decisive in EBV's reactivation process upon challenges imposed to the host cells. In Akata BL cells the strong increase of ZEBRA expression upon BCR cross-linking, the protein coded by *BZLF1*, goes through a bi-phasic process that requires protein synthesis to be amplified [28]. Protein synthesis inhibitors are therefore expected to abrogate the positive feedback loop. In fact, upon BCR cross-linking, we observed lower *BZLF1* mRNA expression levels in cells treated with cycloheximide or 4E1RCat. Interestingly, TLR9 stimulation with ODN CpG 2006 inhibited *BZLF1* mRNA expression at the same ratio, whether protein synthesis was

inhibited or not. This strongly suggests that the regulation of EBV's lytic reactivation upon TLR9 stimulation with ODN CpG 2006 is, to a large extend, protein synthesis independent and suggests post-translational modifications to be responsible for EBV lytic gene repression. The question weather TLR9 activation also affects ZEBRA's activity and the positive amplification ZEBRA exerts on its own promoter remains open. Our group revealed that TLR9 triggering affects histone modifications, which is, at least partly, responsible for EBV lytic gene repression [21]. In addition, several studies showed that epigenetic mechanisms including histone modifications or DNA methylation are responsible for the regulation of EBV lytic gene expression upon BCR cross-linking [32,33]. Consequently, even if we cannot completely exclude the existence of a newly synthesized unknown repressor factor(s), our results strongly indicate that EBV reinforces its latency by hijacking the TLR9-induced post-translational modifications to repress its lytic genes expression.

We unequivocally and unprecedentedly show that MyD88 and IRAK4 are essential for the TLR9-dependent inhibition of EBV lytic reactivation upon BCR cross-linking. This was possible after we inactivated *TLR9*, *MyD88*, or *IRAK4* genes in single cell clones by CRISPR/Cas9 methodology. Importantly, we proved that the canonical TLR9 signaling pathway was completely abrogated by the inactivating mutations as revealed by the lower IL-10 concentrations in the supernatant and the missing NF-κB nuclear translocation after stimulation with ODN CpG 2006. Of note, we excluded that *TLR9, MyD88,* or *IRAK4* inactivation affects the degree of BCR expression on the cell surface. Thus, the TLR9-induced abrogation of the effects subsequent to BCR-cross-linking is not due to changes in BCR expression degree.

Our findings contribute to an increased understanding of the mechanisms involved in the TLR9-induced inhibition of EBV lytic reactivation and confirm our

previously reported observations [21]. Moreover, as reported earlier, TLR9-mediated suppression of EBV lytic gene expression is not restricted to latently infected BL cells but can also be observed *ex vivo* in primary cells upon EBV infection [20]. It seems likely that this feature is shared by other gammaherpesviruses, as we found that stimulation of TLR9, as well as of TLR7, similarly suppresses spontaneous and induced MHV-68 reactivation in mice [34]. In addition, NF-κB nuclear translocation was shown to suppress the lytic genes of the Kaposi's sarcoma-associated herpesvirus [35]. Thus, gammaherpesviruses seem to have evolved to exploit cellular signaling mechanisms in order to keep their latent state and avoid unnecessary aberrant lytic reactivation. The infectious viral particles produced upon EBV lytic reactivation are recognized by the host's immune system and lead to an inflammatory response that could be harmful for both the virus and the host [36]. Our findings indicate that EBV developed a very strong and efficient strategy, by taking advantage of the host cells innate immune TLR9 signaling machinery through MyD88 and IRAK4, to ensure and maintain its reversible latency.

Most intriguingly, we failed to establish *IRAK1* mutated BL cell clones retaining EBV genomes. Since the 3 CRISPR plasmids we developed were efficient in mutating *IRAK1* gene in at least one single cell clone, this preliminary finding suggests that IRAK1 could play a central role in steering EBV persistence. EBV nuclear protein EBNA1 binds to the origin of viral replication and is essential to mediate replication and partitioning of its episome during the division of the host cell. EBNA1 function is regulated by the phosphorylation of ten specific sites [37]. The kinases responsible for EBNA1 phosphorylation have not been identified, but IRAK1 could be a potential candidate. Thus, one hypothesis for the loss of EBV in the *IRAK1*-mutated clones is that EBNA1's function is altered by the inactivation of IRAK1, which affects the replication and

partitioning of EBV's genomic DNA in dividing cells leading to the dilution of the virus in the daughter cells and finally to its loss. However, it is important to consider that these result has only been observed in a limited number of clones, mostly transfected with the IRAK1-b CRISPR/Cas9 plasmid, the most efficient one, in three independent single cell cloning round. The potential importance of IRAK1 activity for EBNA1 function requires the analysis of more clones transfected with different IRAK1- CRISPR/Cas 9 plasmids as well as further investigations.

In summary, our results demonstrate that IRAK4 is essential and very efficient to inhibit EBV lytic reactivation upon TLR9-induction. The TLR9-MyD88-IRAK4 pathway activation favors EBV latency by post-translational modifications and is largely protein synthesis independent. Therefore, the TLR9-MyD88-IRAK4 pathway is a potential therapeutic target to support disruption of EBV latency in EBV-associated lymphoproliferative disorders.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgement

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References

1. Young, L. S. & Rickinson, A. B. Epstein-Barr virus: 40 years on. *Nat. Rev. Cancer* **4,** 757–68 (2004).

2. Speck, S. H. & Ganem, D. Viral latency and its regulation: lessons from the gamma-herpesviruses. *Cell Host Microbe* **8,** 100–15 (2010).

3. Klein, G. Epstein-Barr virus strategy in normal and neoplastic B cells. *Cell* **77,** 791–3 (1994).

4. Brady, G., Macarthur, G. J. & Farrell, P. J. Epstein-Barr virus and Burkitt lymphoma. *Postgrad. Med. J.* **84,** 372–7 (2008).

5. Nemerow, G. R., Mold, C., Schwend, V. K., Tollefson, V. & Cooper, N. R. Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d. *J. Virol.* **61,** 1416–20 (1987).

6. Rowe, D. T. *et al.* Restricted expression of EBV latent genes and T-lymphocyte-detected membrane antigen in Burkitt's lymphoma cells. *EMBO J.* **5,** 2599–607 (1986).

7. Rowe, M., Kelly, G. L., Bell, A. I. & Rickinson, A. B. Burkitt's lymphoma: the Rosetta Stone deciphering Epstein-Barr virus biology. *Semin. Cancer Biol.* **19,** 377–88 (2009).

8. Countryman, J., Jenson, H., Seibl, R., Wolf, H. & Miller, G. Polymorphic proteins encoded within BZLF1 of defective and standard Epstein-Barr viruses disrupt latency. *J. Virol.* **61,** 3672–9 (1987).

9. Tovey, M. G., Lenoir, G. & Begon-Lours, J. Activation of latent Epstein-Barr virus by antibody to human IgM. *Nature* **276,** 270–2 (1978).

10. zur Hausen, H., O'Neill, F. J., Freese, U. K. & Hecker, E. Persisting oncogenic herpesvirus induced by the tumour promotor TPA. *Nature* **272,** 373–5 (1978).

11. Luka, J., Kallin, B. & Klein, G. Induction of the Epstein-Barr virus (EBV) cycle in latently infected cells by n-butyrate. *Virology* **94,** 228–31 (1979).

12. Takada, K. Cross-linking of cell surface immunoglobulins induces Epstein-Barr virus in Burkitt lymphoma lines. *Int. J. Cancer* **33,** 27–32 (1984).

13. di Renzo, L., Altiok, A., Klein, G. & Klein, E. Endogenous TGF-beta contributes to the induction of the EBV lytic cycle in two Burkitt lymphoma cell lines. *Int. J. Cancer* **57,** 914–9 (1994).

14. Lünemann, A., Rowe, M. & Nadal, D. in (ed. Münz, C.) 265–287 (Springer International Publishing, 2015). doi:10.1007/978-3-319-22834-1_9

15. Middeldorp, J. M. in (ed. Münz, C.) 289–323 (Springer International Publishing, 2015). doi:10.1007/978-3-319-22834-1_10

16. Hislop, A. D. & Taylor, G. S. in (ed. Münz, C.) 325–353 (Springer International Publishing, 2015). doi:10.1007/978-3-319-22834-1_11

17. Murata, T. *et al.* Epigenetic histone modification of Epstein-Barr virus BZLF1 promoter during latency and reactivation in Raji cells. *J. Virol.* **86,** 4752–61 (2012).

18. Ramasubramanyan, S., Osborn, K., Flower, K. & Sinclair, A. J. Dynamic chromatin environment of key lytic cycle regulatory regions of the Epstein-Barr virus genome. *J. Virol.* **86,** 1809–19 (2012).

19. Bryant, H. & Farrell, P. J. Signal Transduction and Transcription Factor Modification during Reactivation of Epstein-Barr Virus from Latency. *J. Virol.* **76,** 10290–8 (2002).

20. Ladell, K. *et al.* Immune activation suppresses initiation of lytic Epstein-Barr virus infection. *Cell. Microbiol.* **9,** 2055–69 (2007).

21. Zauner, L. *et al.* TLR9 triggering in Burkitt's lymphoma cell lines suppresses the EBV BZLF1 transcription via histone modification. *Oncogene* **29,** 4588–98 (2010).

22. Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* **11,** 373–84 (2010).

23. Takeuchi, O. & Akira, S. Pattern recognition receptors and inflammation. *Cell* **140,** 805–20 (2010).

24. Heeg, K., Dalpke, A., Peter, M. & Zimmermann, S. Structural requirements for uptake and recognition of CpG oligonucleotides. *Int. J. Med. Microbiol.* **298,** 33–8 (2008).

25. Takeshita, F. *et al.* Signal transduction pathways mediated by the interaction of CpG DNA with Toll-like receptor 9. *Semin. Immunol.* **16,** 17–22 (2004).

26. Rechsteiner, M. P. *et al.* Latent membrane protein 2B regulates susceptibility to induction of lytic Epstein-Barr virus infection. *J. Virol.* **82,** 1739–47 (2008).

27. Coban, C. *et al.* Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J. Exp. Med.* **201,** 19–25 (2005).

28. Flemington, E. K., Goldfeld, A. E. & Speck, S. H. Efficient transcription of the Epstein-Barr virus immediate-early BZLF1 and BRLF1 genes requires protein synthesis. *J. Virol.* **65,** 7073–7 (1991).

29. Yin, Q., Jupiter, K. & Flemington, E. K. The Epstein-Barr virus transactivator Zta binds to its own promoter and is required for full promoter activity during anti-Ig and TGF-beta1 mediated reactivation. *Virology* **327,** 134–43 (2004).

30. Ye, J., Gradoville, L. & Miller, G. Cellular immediate-early gene expression occurs

kinetically upstream of Epstein-Barr virus bzlf1 and brlf1 following cross-linking of the B cell antigen receptor in the Akata Burkitt lymphoma cell line. *J. Virol.* **84,** 12405–18 (2010).

31. Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8,** 2281–308 (2013).

32. Jenkins, P. J., Binné, U. K. & Farrell, P. J. Histone acetylation and reactivation of Epstein-Barr virus from latency. *J. Virol.* **74,** 710–20 (2000).

33. Countryman, J. K., Gradoville, L. & Miller, G. Histone hyperacetylation occurs on promoters of lytic cycle regulatory genes in Epstein-Barr virus-infected cell lines which are refractory to disruption of latency by histone deacetylase inhibitors. *J. Virol.* **82,** 4706–19 (2008).

34. Haas, F. *et al.* Activation of NF-κB via endosomal Toll-like receptor 7 (TLR7) or TLR9 suppresses murine herpesvirus 68 reactivation. *J. Virol.* **88,** 10002–12 (2014).

35. Izumiya, Y. *et al.* NF-kappaB serves as a cellular sensor of Kaposi's sarcoma-associated herpesvirus latency and negatively regulates K-Rta by antagonizing the RBP-Jkappa coactivator. *J. Virol.* **83,** 4435–46 (2009).

36. Callan, M. F. C. The immune response to Epstein-Barr virus. *Microbes Infect.* **6,** 937–45 (2004).

37. Duellman, S. J., Thompson, K. L., Coon, J. J. & Burgess, R. R. Phosphorylation sites of Epstein-Barr virus EBNA1 regulate its function. *J. Gen. Virol.* **90,** 2251–9 (2009).

Figure Legends

Figure 1: Experimental design for the establishment of CRISPR/Cas9 single cell clones. Steps for reagent design, construction, cell line expansion and characterization are depicted. Three different custom sgRNAs (light blue bars) were designed *in silico* via the CRISPR Design Tool (http://crispr.mit.edu/). sgRNA guide sequences were cloned into the expression plasmid pSpCas9(BB)-2A-GFP (PX458) bearing sgRNA scaffold backbone (BB), Cas9, and GFP. Cloned and sequence-verified pSpCas9(BB)-2A-GFP (PX458) plasmids were then electroporated into Akata cells, after 48h, GFP-positive cells were single cell sorted by FACS. Finally, the GFP positive single cell clones were expanded, genotyped and characterized. (Adapted from [31])

Figure 2: TLR9 triggering via ODN CpG 2006 inhibits EBV master lytic gene *BZLF1* mRNA expression in BL cells despite protein synthesis inhibition with cycloheximide and 4E1RCat.

(**a**) Akata cells were treated with the protein synthesis inhibitors cycloheximide (33 µg/ml) or 4E1RCat (10 µM and 25 µM), for 8.5h. Cell viability was determined by Trypan blue exclusion assay. Untreated and vehicle-treated cells (H_2O for cycloheximide and DMSO for 4E1RCat) were used as negative controls; digitonin (30 µg/ml) treated cells as a positive control for cell toxicity after 8.5h incubation. Results are shown as percentage of viable cells (n=3). (**b**) and (**c**) Protein synthesis inhibitors cycloheximide (33 µg/ml) and 4E1RCat (10 µM), were added first to the Akata cells. After 30 min incubation, cells were treated for 2h with ODN CpG 2006, and finally for 6h with anti-IgG. After a total incubation time of 8.5h the cells were lysed and the mRNA extracted. The *BZLF1* mRNA level relative to *HMBS* was measured by RT-qPCR. (n=3). (**b**) Represents an enlargement of the *BZLF1* mRNA levels measured upon incubation with ODN CpG 2006,

cycloheximide or 4E1RCat only, which is presented in (**c**). Data are represented as mean ± SD. Statistics were calculated using the unpaired *t* test. (*, $P<0.05$)

Figure 3: CRISPR/Cas9 mutation of TLR9, MyD88, IRAK4 and IRAK1 result in a complete TLR9 signaling abrogation, and affect EBV viral copy numbers per cells. Akata Burkitt's lymphoma B cells transfected with px458 plasmids coding for Cas9 and containing a sgRNA targeting TLR9 (TLR9-b3, TLR9-b5 and TLR9-b6), MyD88 (MyD88-a3, MyD88-a5 and MyD88-b1), IRAK4 (IRAK4-a3 and IRAK4-a4) and IRAK1 (IRAK1-b2 and IRAK1-b8), respectively, were diluted for single cell cloning, sequenced and clones containing an early stop codon were selected for further characterization. (**a**) % of IgG positive cells was measured by flow cytometry. (**b**) *TLR9* mRNA level was measured by RT-qPCR and normalized over *HMBS* and over WT Akata cells. (**c**) IL-10 cytokine expression level measured by ELISA in the supernatant of untreated cells, and cells treated for 8h ODN CpG 2006. (**d**) Viral *BamH1 W* copy numbers over cellular *HMBS* determined by qPCR. (**a**) Has been performed only once (**b, c, d**) Shows one representative experiment out of three. Data are represented as mean ± SD

Figure 4: TLR9, MyD88 or IRAK4 inactivation inhibits NF-κB nuclear translocation upon TLR9 activation with ODN CpG 2006.
Results show NF-κB p65 protein expression in the cytoplasmic and nuclear fraction of Akata WT cells and TLR9 (TLR9-b3, TLR9-b5 and TLR9-b6), MyD88 (MyD88-a3, MyD88-a5 and MyD88-b1) and IRAK4 (IRAK4-a3 and IRAK4-a4) CRISPR single cell clones. Cells were treated with ODN CpG 2006 for 90'. Lamin A/C and tubulin protein expressions were measured as loading control for the cytoplasmic respectively nuclear fractions. One representative experiment is shown out of three independent experiments.

Figure 5: TLR9-induced inhibition of *BZLF1* expression is strictly TLR9, MyD88 and IRAK4 dependent.

CRISPR/Cas9 mutated TLR9, MyD88 and IRAK4 clones were treated for 2h with ODN CpG 2006 prior to lytic reactivation by Ig-crosslinking with anti-IgG for 6h. *BZLF1* mRNA expression level normalized to *HMBS* was measured by RT-qPCR and relative over untreated cells in TLR9 clones, MyD88 clones and IRAK4 clones. Shown is one representative experiment out of three. Data are represented as mean ± SD (n=3). Statistics were calculated using the unpaired *t* test. (***, $P<0.001$; *, $P<0.05$; n.s., not significant).

Table 1: CRISPR single guide RNA target sequences.

Target name	Genomic locus	Target Sequence (5'-3')
IRAK4$^-$ a	IRAK4 exon 2	AGGCAGCGCACATATGTTGATGG
IRAK4$^-$ b	IRAK4 exon 2	TATGTGCGCTGCCTCAATGTTGG
IRAK4$^-$ c	IRAK4 exon 2	GCCTCAATGTTGGACTAATTAGG
IRAK1$^-$ a	IRAK1 exon 2	CGGTCTGGTCGCGCACGATCAGG
IRAK1$^-$ b	IRAK1 exon 2	GATCAACCGCAACGCCCGTGTGG
IRAK1$^-$ c	IRAK1 exon 2	GGTCTGGTCGCGCACGATCAGGG
MyD88$^-$ a	MyD88 exon 1	GTTCTTGAACGTGCGGACACAGG
MyD88$^-$ b	MyD88 exon 1	GCTCCAGCAGCACGTCGTCGCGG
MyD88$^-$ c	MyD88 exon 3	ATGAAGGCATCGAAACGCTCAGG
TLR9$^-$ a	TLR9 exon 2	CGCTGATGCGGTTGTCCGACAGG
TLR9$^-$ b	TLR9 exon 2	ACTGGGTGTACAACGAGCTTCGG
TLR9$^-$ c	TLR9 exon 2	GCTCACGGCTATTCGGCCGTGGG

Table 2: Sequencing summary of CRISPR/Cas9 IRAK4$^-$, TLR9$^-$ and MyD88$^-$ clones.

Clones	DNA sequence changes	Amino acid change
IRAK4$^-$ a3	c.25inA	p.Thr9AspfsX19
IRAK4$^-$ a4	c.24delA	p.Thr9HisX17
TLR9$^-$ b3	c.2615_2690delCCTTCGTGGTCTTCGACAAAACGCAGAGC GCAGTGGCAGACTGGGTGTACAACGAGCTTCGGGGGCA GCTGGAGGA + c.2692T>C	p.Ala872GlyX945
TLR9$^-$ b5	c.2672inC	p.Leu891ProfsX921
TLR9$^-$ b6 1*	c.2672inC	p.Leu891ProfsX921
TLR9$^-$ b6 2*	c.2655_2682delCTGGGTGTACAACGAGCTTCGGGGGCAG	p.Val887ArgfsX960
MyD88$^-$ a3	c.153_165delGTGCGGACACAG	p.Arg53AlafsX122
MyD88$^-$ a5	c.161delC	p.Thr54AspfsX99
MyD88$^-$ b1	c.336delC	p.Asp112GlufsX273

DNA sequence changes nomenclature is based on the coding DNA sequence. * TLR9$^-$ b6 derives from two different clones whose sequence was determined by TA cloning and sequencing.

Table 3: Summary of the CRISPR Cas9 transfection and characterization experiments.

	IRAK1$^-$	IRAK4$^-$	TLR9$^-$	MyD88$^-$	Akata px458-ctl
Inactive TLR9 pathway (ko)	13/23[a]	4/10[a]	5/10[a]	6/10[a]	0/12[a]
EBV positive	7/23[b]	6/10[b]	6/10[b]	9/10[b]	10/12[b]
EBV positive / TLR9 ko	0/13[c]	3/4[c]	3/5[c]	5/6[c]	0/0[c]

[a] = number of single cell clones with an inactive TLR9 pathway over the total number of single cell clones analyzed; [b] = number of EBV positive single cell clones over the total number of single cell clones analyzed; [c] = number of EBV positive single cell clones over the number of single cell clones with an inactive TLR9 pathway.

Figure 1:

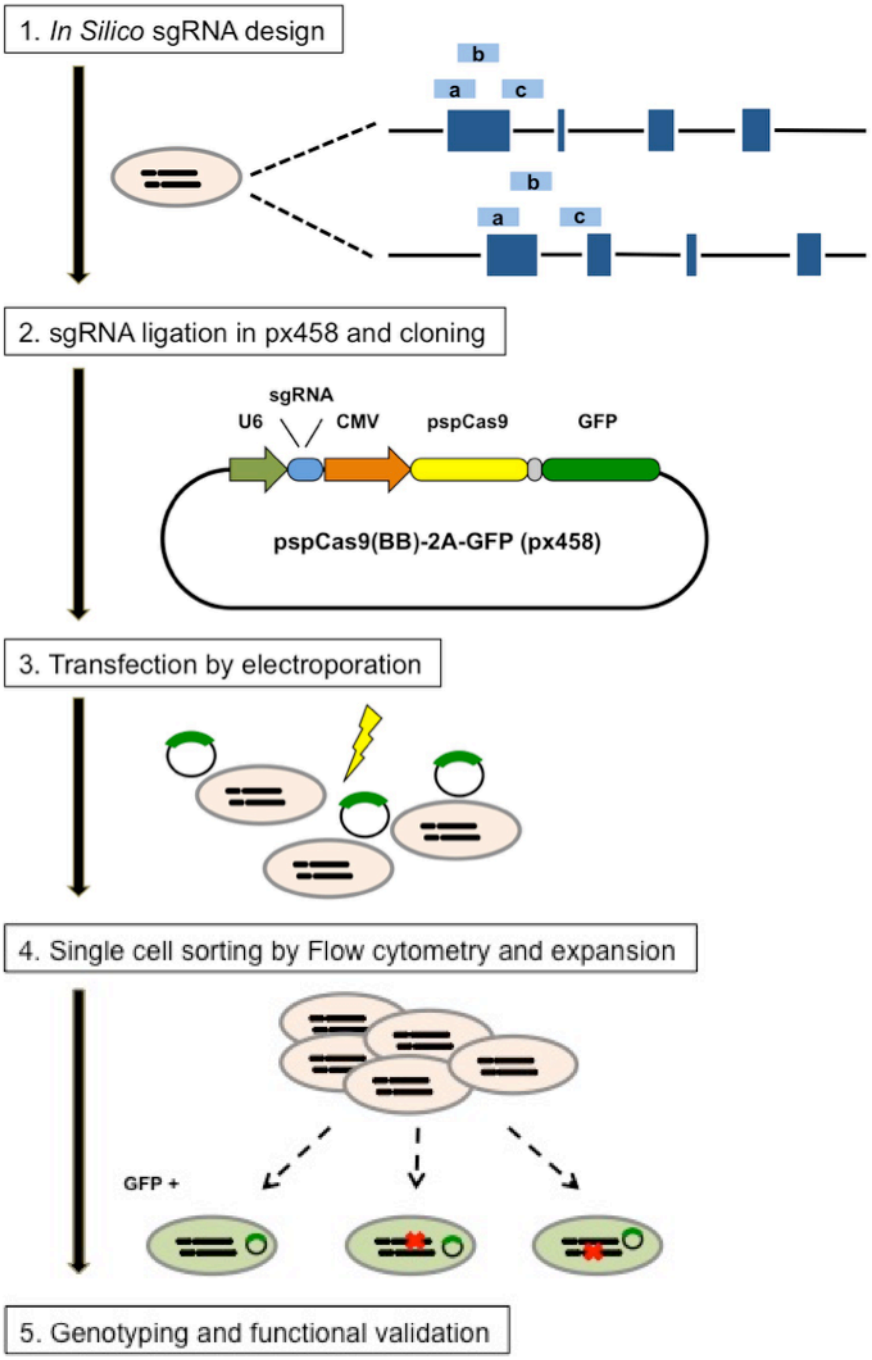

1. In Silico sgRNA design
b
a
c
b
a
c
2. sgRNA ligation in px458 and cloning
sgRNA
U6
CMV
pspCas9
GFP
pspCas9(BB)-2A-GFP (px458)
3. Transfection by electroporation
4. Single cell sorting by Flow cytometry and expansion
GFP +
5. Genotyping and functional validation

Figure 2:

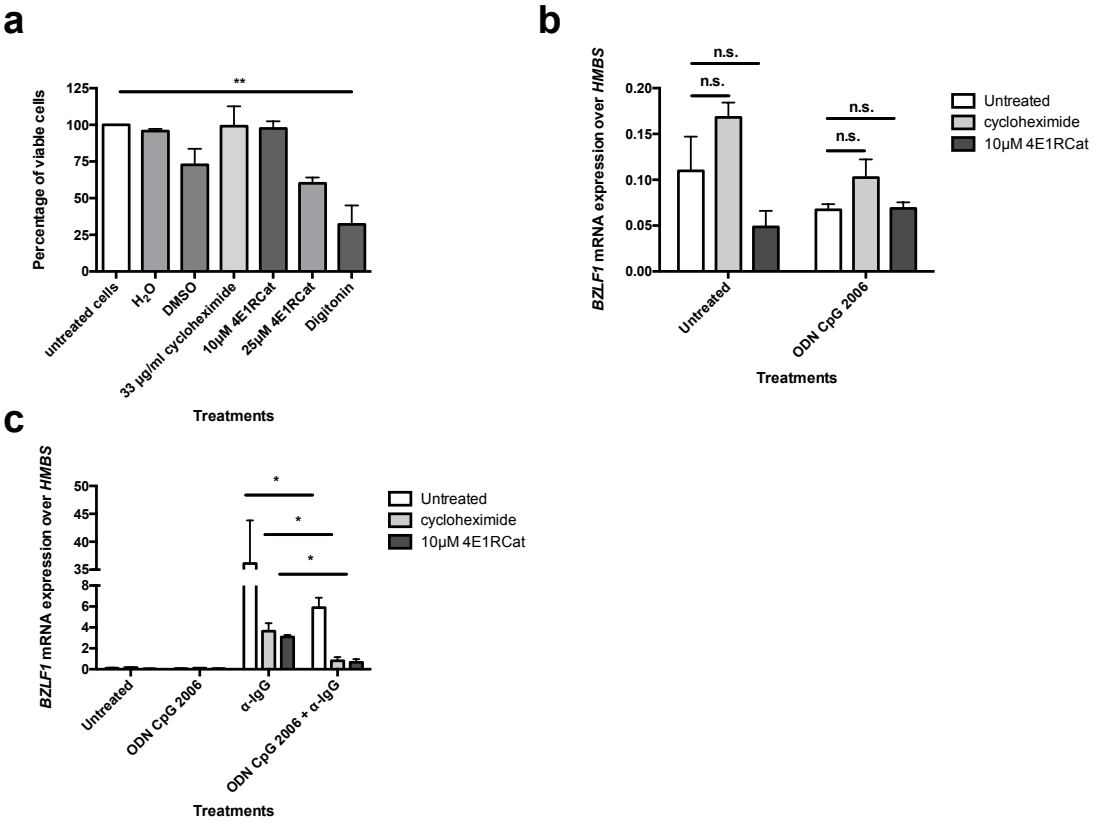

a
Percentage of viable cells
**
untreated cells
H2O
DMSO
33 µg/ml cycloheximide
10µM 4E1RCat
25µM 4E1RCat
Digitonin
Treatments
b
BZLF1 mRNA expression over HMBS
n.s.
n.s.
n.s.
n.s.
Untreated
cycloheximide
10µM 4E1RCat
Untreated
ODN CpG 2006
Treatments
c
BZLF1 mRNA expression over HMBS
*
*
*
Untreated
cycloheximide
10µM 4E1RCat
Untreated
ODN CpG 2006
α-IgG
ODN CpG 2006 + α-IgG
Treatments

Figure 3:

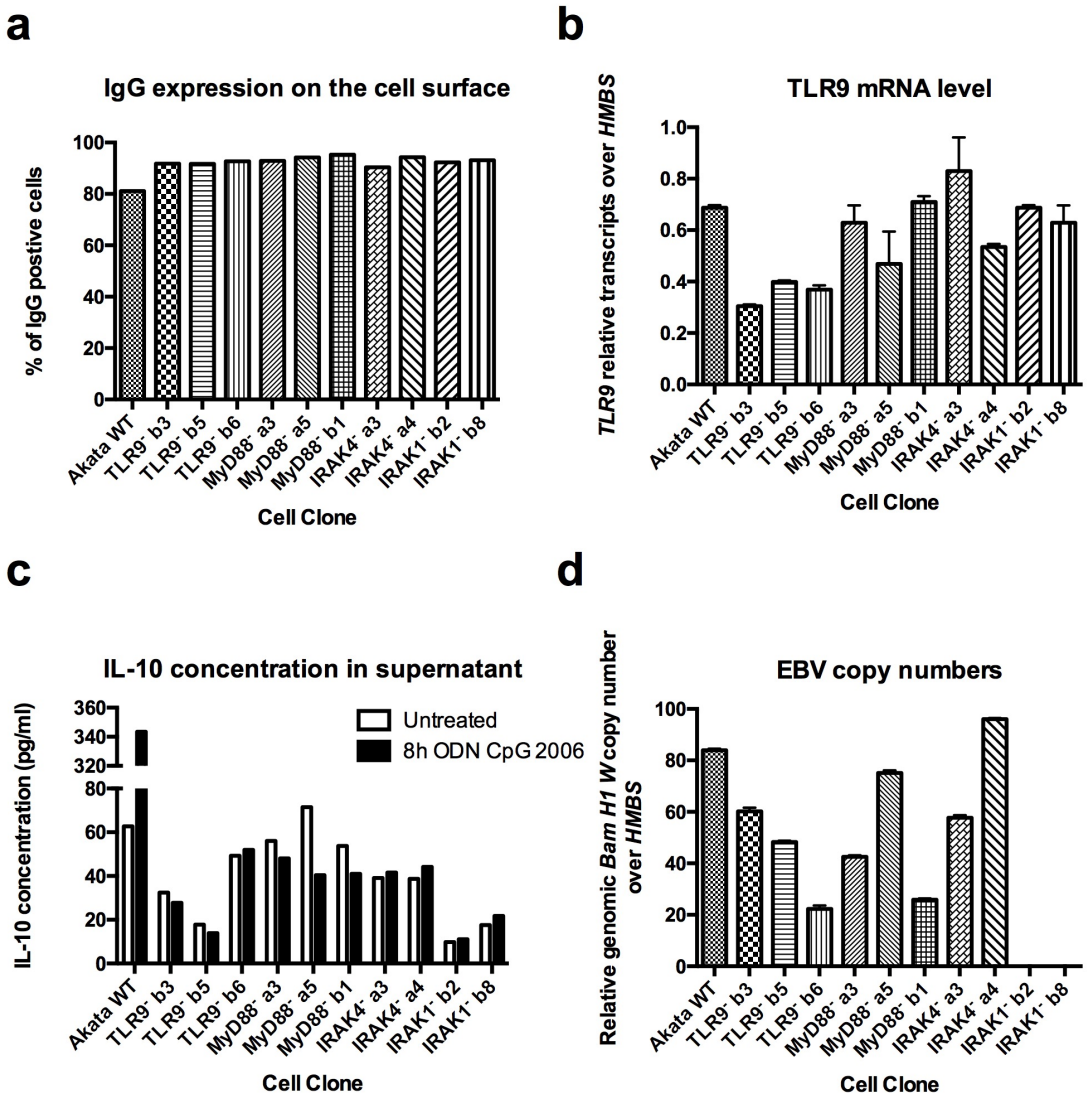

a
IgG expression on the cell surface
% of IgG postive cells
100
80
60
40
20
0
Akata WT
TLR9- b3
TLR9- b5
TLR9- b6
MyD88- a3
MyD88- a5
MyD88- b1
IRAK4- a3
IRAK4- a4
IRAK1- b2
IRAK1- b8
Cell Clone
b
TLR9 mRNA level
TLR9 relative transcripts over HMBS
1.0
0.8
0.6
0.4
0.2
0.0
Akata WT
TLR9- b3
TLR9- b5
TLR9- b6
MyD88- a3
MyD88- a5
MyD88- b1
IRAK4- a3
IRAK4- a4
IRAK1- b2
IRAK1- b8
Cell Clone
c
IL-10 concentration in supernatant
IL-10 concentration (pg/ml)
360
340
320
80
60
40
20
0
Untreated
8h ODN CpG 2006
Akata WT
TLR9- b3
TLR9- b5
TLR9- b6
MyD88- a3
MyD88- a5
MyD88- b1
IRAK4- a3
IRAK4- a4
IRAK1- b2
IRAK1- b8
Cell Clone
d
EBV copy numbers
Relative genomic Bam H1 W copy number over HMBS
100
80
60
40
20
0
Akata WT
TLR9- b3
TLR9- b5
TLR9- b6
MyD88- a3
MyD88- a5
MyD88- b1
IRAK4- a3
IRAK4- a4
IRAK1- b2
IRAK1- b8
Cell Clone

Figure 4:

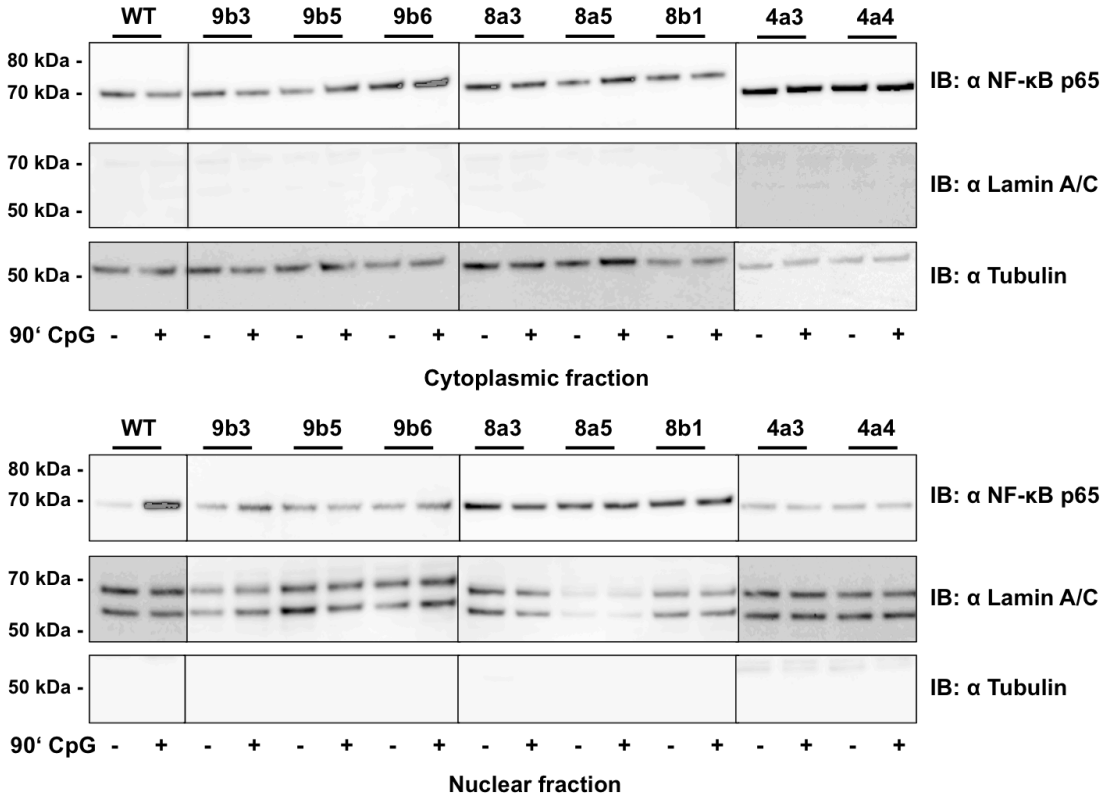
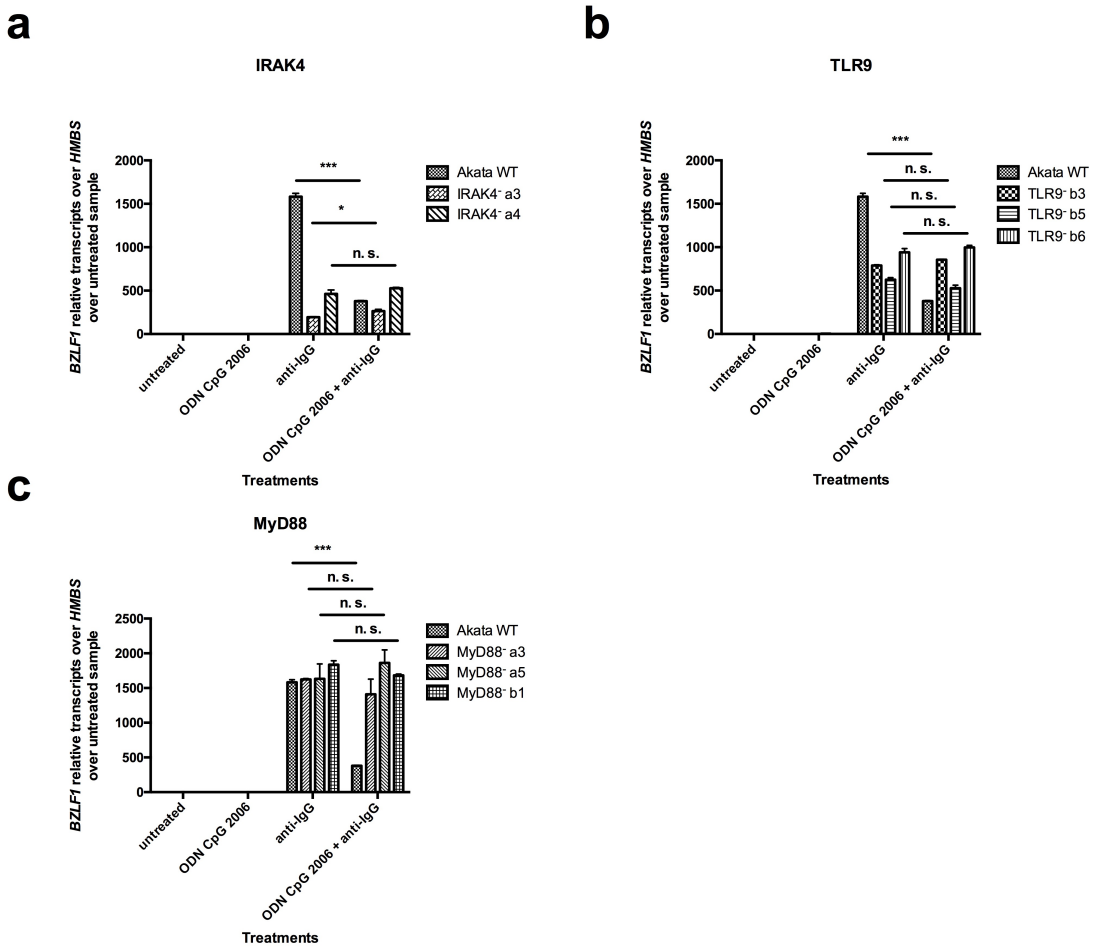
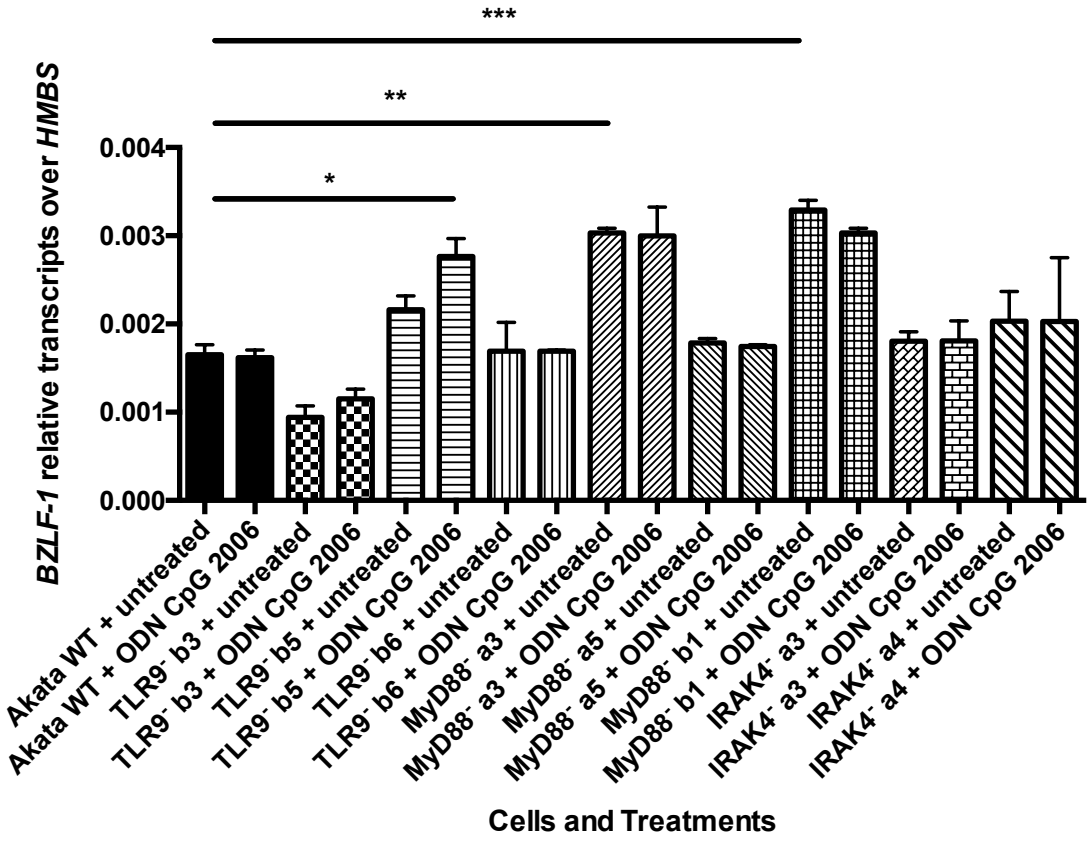

WT
9b3
9b5
9b6
8a3
8a5
8b1
4a3
4a4
80 kDa -
70 kDa -
IB: α NF-κB p65
70 kDa -
50 kDa -
IB: α Lamin A/C
50 kDa -
IB: α Tubulin
90‘ CpG - + - + - + - + - + - + - + - + - +
Cytoplasmic fraction
WT
9b3
9b5
9b6
8a3
8a5
8b1
4a3
4a4
80 kDa -
70 kDa -
IB: α NF-κB p65
70 kDa -
50 kDa -
IB: α Lamin A/C
50 kDa -
IB: α Tubulin
90‘ CpG - + - + - + - + - + - + - + - + - +
Nuclear fraction

Figure 5:


a
IRAK4
BZLF1 relative transcripts over HMBS over untreated sample
2000
1500
1000
500
0
***
*
n. s.
Akata WT
IRAK4- a3
IRAK4- a4
untreated
ODN CpG 2006
anti-IgG
ODN CpG 2006 + anti-IgG
Treatments
b
TLR9
BZLF1 relative transcripts over HMBS over untreated sample
2000
1500
1000
500
0
***
n. s.
n. s.
n. s.
Akata WT
TLR9- b3
TLR9- b5
TLR9- b6
untreated
ODN CpG 2006
anti-IgG
ODN CpG 2006 + anti-IgG
Treatments
c
MyD88
BZLF1 relative transcripts over HMBS over untreated sample
2500
2000
1500
1000
500
0
***
n. s.
n. s.
n. s.
Akata WT
MyD88- a3
MyD88- a5
MyD88- b1
untreated
ODN CpG 2006
anti-IgG
ODN CpG 2006 + anti-IgG
Treatments

Supplementary Figure S1:

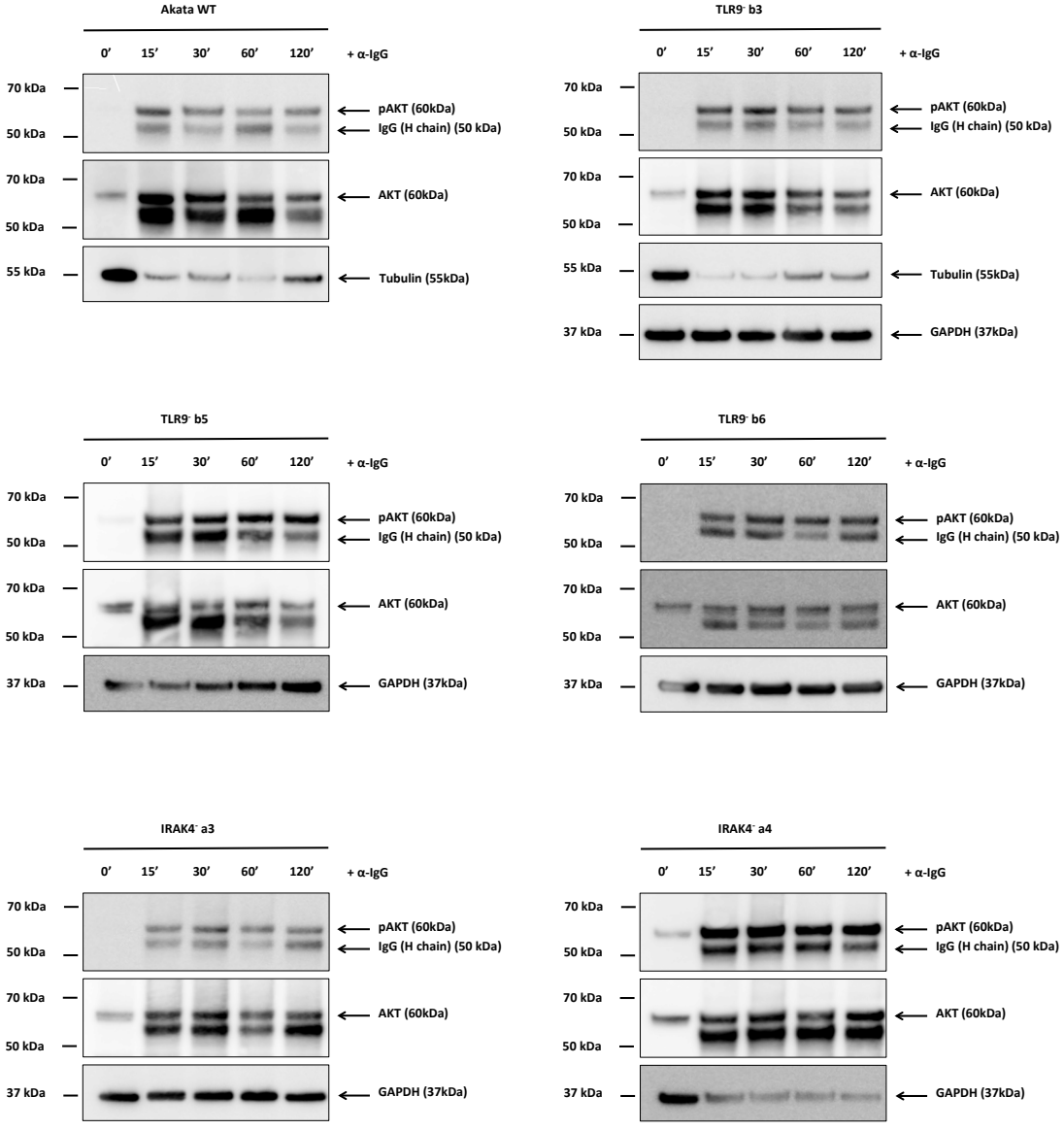
CRISPR/Cas9 mutated TLR9, MyD88 and IRAK4 clones were treated for 2h with ODN CpG 2006. *BZLF1* mRNA expression level normalized to *HMBS* was measured by RT-qPCR in TLR9$^-$ clones, MyD88$^-$ clones and IRAK4$^-$ clones. Shown is one representative experiment out of three. Data are represented as mean ± SD (n=3). Statistics were calculated using the unpaired *t* test. (***, *P*<0.001; *, *P*<0.05; n.s., not significant).



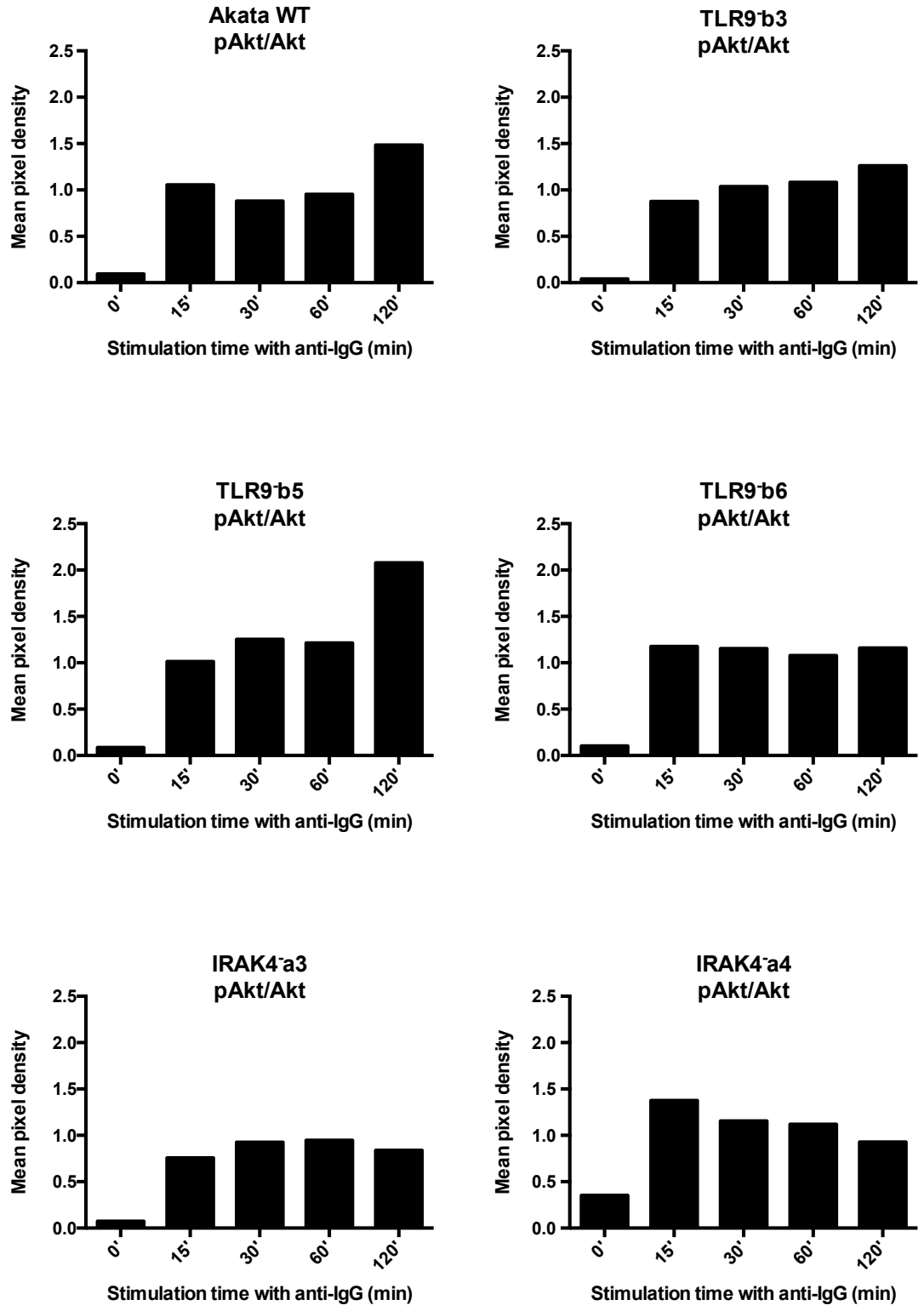
Supplementary Figure S2:

Akata WT cells and TLR9 (TLR9-b3, TLR9-b5 and TLR9-b6) and IRAK4 (IRAK4-a3 and IRAK4-a4) CRISPR/Cas9 single cell clones were treated with ODN CpG 2006 for 15', 30', 60' and 120'. (**a**) Results show pAkt and Akt protein expression. GAPDH or Tubulin protein expressions were measured as loading control. (**b**) Graphical representation of the densitometric measurement of (**a**). pAkt values were calculated relatively to the Akt level. One representative experiment is shown.

a



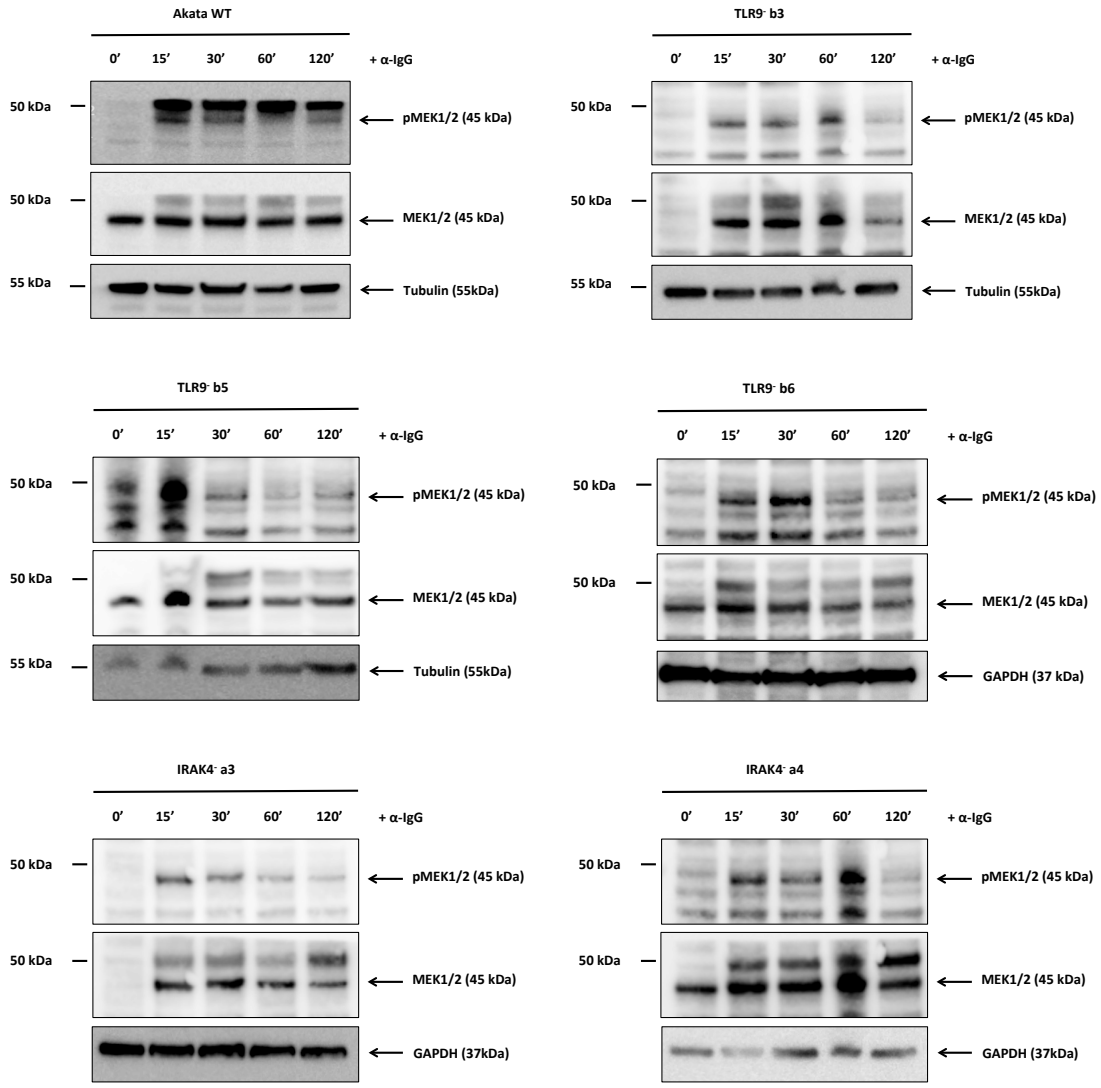
b


Akata WT
pAkt/Akt
TLR9-b3
pAkt/Akt
TLR9-b5
pAkt/Akt
TLR9-b6
pAkt/Akt
IRAK4-a3
pAkt/Akt
IRAK4-a4
pAkt/Akt
Mean pixel density
0.0
0.5
1.0
1.5
2.0
2.5
0'
15'
30'
60'
120'
Stimulation time with anti-IgG (min)

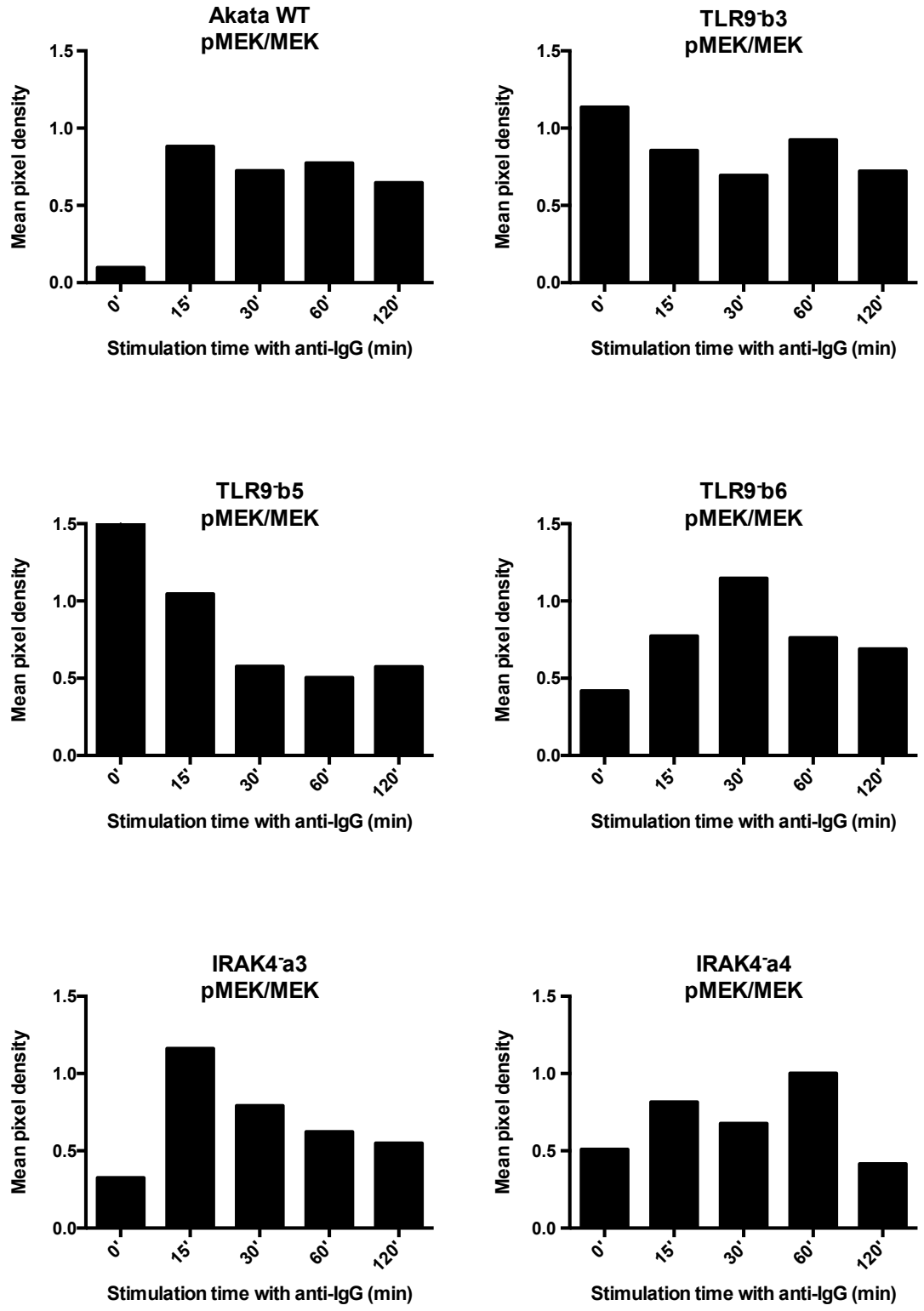
Supplementary Figure S3:

Akata WT cells and TLR9 (TLR9-b3, TLR9-b5 and TLR9-b6) and IRAK4 (IRAK4-a3 and IRAK4-a4) CRISPR/Cas9 single cell clones were treated with ODN CpG 2006 for 15', 30', 60' and 120'. (**a**) Results show pMek1/2 and Mek1/2 protein expression. GAPDH or Tubulin protein expressions were measured as loading control. (**b**) Graphical representation of the densitometric measurement of (**a**). pMek1/2 values were calculated relatively to the Mek1/2 level. One representative experiment is shown.

a



b


Akata WT
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TLR9-b3
pMEK/MEK
TLR9-b5
pMEK/MEK
TLR9-b6
pMEK/MEK
IRAK4-a3
pMEK/MEK
IRAK4-a4
pMEK/MEK
Mean pixel density
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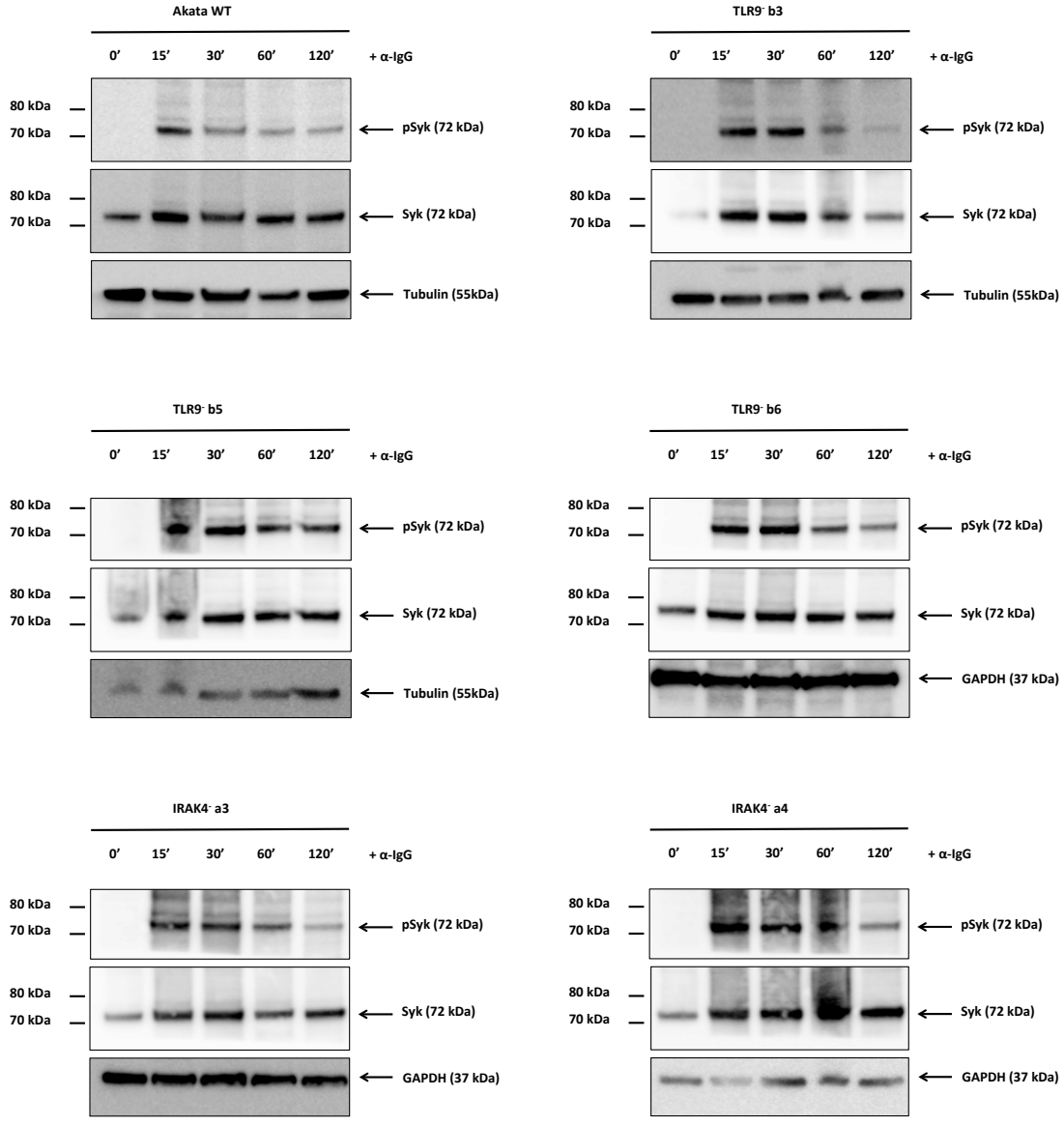
1.0
0.5
0.0
0'
15'
30'
60'
120'
Stimulation time with anti-IgG (min)

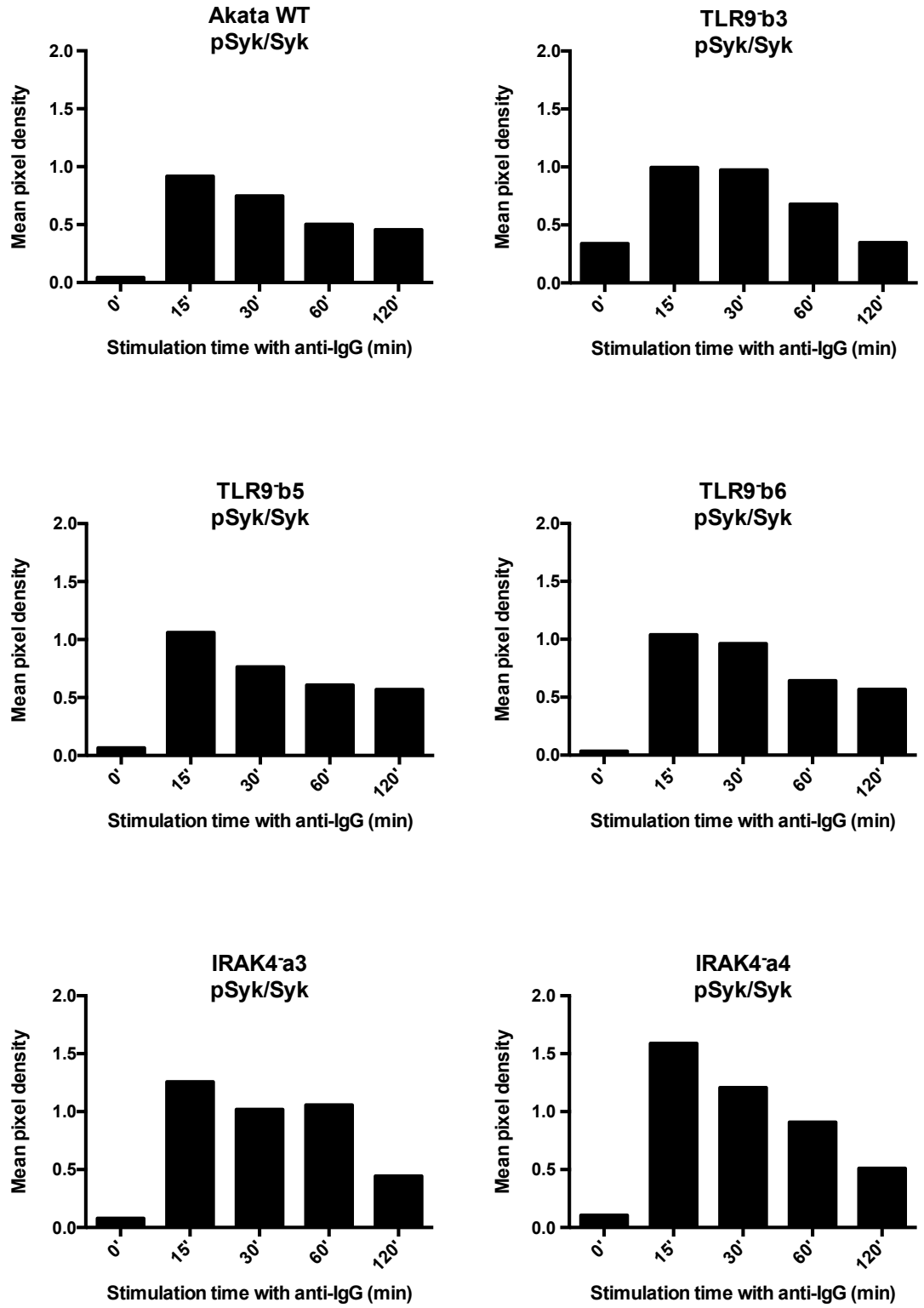
Supplementary Figure S4:

Akata WT cells and TLR9 (TLR9-b3, TLR9-b5 and TLR9-b6) and IRAK4 (IRAK4-a3 and IRAK4-a4) CRISPR/Cas9 single cell clones were treated with ODN CpG 2006 for 15', 30', 60' and 120'. (**a**) Results show pSyk and Syk protein expression. GAPDH or Tubulin protein expressions were measured as loading control. (**b**) Graphical representation of the densitometric measurement of (**a**). pSyk values were calculated relatively to the Syk level. One representative experiment is shown.

a



b


Akata WT
pSyk/Syk
TLR9-b3
pSyk/Syk
TLR9-b5
pSyk/Syk
TLR9-b6
pSyk/Syk
IRAK4-a3
pSyk/Syk
IRAK4-a4
pSyk/Syk
Mean pixel density
2.0
1.5
1.0
0.5
0.0
0'
15'
30'
60'
120'
Stimulation time with anti-IgG (min)

Manuscript II:

TLR9 induction suppresses Epstein-Barr virus lytic reactivation in Burkitt's lymphoma cells by modifying histones structure on the genomic *Zp* promoter but not on reporter systems

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Abstract

Background: The triggering of Toll-like receptor 9 (TLR9) results in the suppression of EBV's master regulatory lytic gene *BZLF1*. The reduction of EBV lytic gene expression promotes viral latency in the cells and latent EBV, in turn, might enhance the possibility for cell transformation. Lytic reactivation of EBV is associated with modification of chromatin structure on the *BZLF1* promoter *Zp*.

Results: To characterize the effect of TLR9 on the *BZLF1* promoter *Zp* in more detail, we evaluated the TLR9-induced suppression of lytic EBV for different *Zp* regions coupled to a luciferase gene in a reporter system. Unexpectedly, TLR9 triggering inhibited *BZLF1* expression in BCR-induced Akata Burkitt's lymphoma cells latently infected with EBV, but did not affect the activation of *Zp* reporter constructs. TLR9-induced histone modifications observed in genomic EBV upon lytic reactivation were not found in episomal reporter constructs.

Conclusions: The underlying mechanism of the differential effect of TLR9 signaling might mirror the distinct chromatin structure of the transfected *Zp* reporter construct compared to EBV's viral genome. This reporter system based research does not reflect the viral reality and other strategies should be considered to study EBV's lytic reactivation in order to use it for cancer cell-tailored therapeutic approach.

Keywords: Innate immunity, CpG, TLR9, Epstein-Barr virus, EBV, Burkitt's lymphoma

Background

Epstein-Barr virus (EBV) is a B-lymphotropic DNA gamma-herpesvirus that persists benignly in a latent state in over 90 % of the world's adult population. EBV's genome is maintained as an episome in latently infected cells [1]. Intermittently, an appropriate stimulus leads to the induction of EBV lytic replication, resulting in the production of new EBV particles and death of the host cell [2].

Latent state EBV is associated with B-cell malignancies including Burkitt's lymphoma (BL) [3], the endemic form of which is epidemiologically linked to *Plasmodium falciparum* malaria [4]. Based on the lethal effect of lytic EBV for the host cell, induction of lytic EBV is being explored as a specific cancer cell-targeted therapy for EBV-associated malignancies [5].

The physiological stimuli that control the switch from latency to lytic EBV *in vivo* are not known [6]. The study of EBV reactivation *in vivo* or *ex vivo* is hampered by the low abundance of B-cells carrying latent EBV. Thus, BL-derived cell lines are used as model to study the switch from EBV latency to lytic reactivation [7, 8]. Various agents including 12-O-tetradecanoylphorbol-13-acetate [9], the cytokine transforming growth factor-β [10], and B-cell receptor (BCR) cross-linking antibodies [11] induce EBV lytic reactivation by triggering cellular signaling pathways resulting in the induction of EBV's immediate-early lytic gene *BZLF1,* which is essential for reactivation of EBV [12].

We have shown that induction of EBV lytic reactivation in BL cells can be suppressed by triggering B-cell Toll-like receptor (TLR)9 *in vitro* [13] and that TLR9-mediated suppression of *BZLF1* involves chromatin remodeling impeding activation of the immediate-early lytic gene [14]. TLRs are transmembrane receptors that sense microbes, and the interaction of a TLR with its pathogen-associated molecular pattern mediates an intracellular signal that results in the production of chemical mediators and cell surface molecules directing innate and acquired immune responses [15]. Endosome-located TLR9, highly expressed by all developmental stages of mature B-cells [16], recognizes pathogen DNA, which contains

unmethylated cytosine-phosphatidyl-guanosine (CpG) motifs [17] and *P. falciparum* pigment hemozoin [18], and can also be triggered by synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG [19]. TLR9 signaling favors EBV latency and likely promotes EBV-associated B-cell lymphoma by counteracting induction of EBV lytic gene expression.

The control of the *Zp* promoter is a key step in the switching from latency to lytic infection [7, 20]. *BZLF1* encodes the transcription factor Zta, and Zta binds specific target sequences in the promoters of EBV's early lytic genes, but also to its own promoter *Zp*. This auto activation of *BZLF1* expression appears to be the basis for EBV's efficient switch to lytic infection [21]. The Zta minimal promoter region *Zp*-221 is known to harbor the elements for maintaining low basal activity and for the transcriptional activation by agents inducing lytic EBV infection [9, 21]. Notably, upstream of -221 bp several negative elements have been identified [22, 23]. Nevertheless, the *Zp* promoter regions affected upon TLR9 triggering remain unknown. A detailed understanding of the regulation of the *Zp* promoter could offer new insights in mechanisms contributing to securing EBV's latency, potentially promoting EBV-associated B-cell lymphomas, and also to devise anti-cancer treatments aiming at the induction of lytic EBV.

Here, we aimed to further elucidate the mechanism leading to TLR9 triggering-induced suppression of EBV lytic reactivation. Thus, we transfected Akata BL cells with various episomal *Zp* reporter constructs and measured the activation of the *Zp* promoter in response to TLR9 triggering and subsequent BCR cross-linking. Our study demonstrates that TLR9 triggering-induced suppression of EBV lytic reactivation is dependent on inhibition/reversal of H3K9 acetylation on EBV genomic *BZLF1* promoter induced by BCR-signaling. This has implications on treatment modalities of EBV-associated cancers based on lytic reactivation of EBV.

Results and discussion

The *Zp*-221 region of the reporter construct is alone sufficient for the induction of EBV lytic gene expression mediated by BCR cross-linking

Triggering of TLR9 results in the suppression of EBV *BZLF1* lytic gene expression [13, 14]. The region of the *BZLF1* promoter *Zp* that is affected by TLR9 signaling leading to suppression of *BZLF1* expression is unknown. We aimed at elucidating the affected region by using *Zp* reporter constructs. To this end, we first validated the function of the *BZLF1* reporter constructs by co-transfection of HeLa cells with the pHEBo-221 *Zp* luciferase plasmid and the *BZLF1* pLenti4 plasmid, which constitutively expresses the transcription factor Zta. Constitutively expressed Zta binds to the *BZLF1* promoter region in the reporter constructs and therefore induces expression of luciferase [24]. By increasing the amount of transfected pHEBo-221 *Zp* luciferase plasmid, we detected a striking augmentation of luciferase expression (Supplementary Figure 1). Thus, we confirmed functionality of *BZLF1* reporter constructs.

To identify the promoter region responsible for the TLR9 triggering-mediated inhibition of lytic EBV, we stably transfected Akata BL cells with various reporter constructs including sequences of varying length of the *BZLF1* promoter: pHEBo-221 *Zp* luciferase, pHEBo-554 *Zp* luciferase pHEBo-900 *Zp* luciferase. Experiments with pHEBo-2.3Kb Zp luciferase were performed as transient transfections, since, despite our efforts, it was not possible to select for Akata cells stably transfected with the pHEBo-2.3Kb *Zp* luciferase construct. We confirmed successful transfection of Akata BL cells using a sequence specific PCR targeting the pHEBO-221 *Zp* luciferase region (data not shown). Additionally, we used the pHEBo luciferase plasmid without a *BZLF1* promoter as a negative control. BCR cross-linking on transfected Akata BL cells led to a significant increase in luciferase expression in pHEBo-221 *Zp* luciferase, pHEBo-554 *Zp* luciferase pHEBo-900 *Zp* luciferase and pHEBo-2.3Kb *Zp* luciferase but not in pHEBo luciferase transfected Akata cells (Figure 1). Akata BL cells

transiently transfected with the different *BZLF1* reporter constructs showed similar results (data not shown). Our data confirmed that the short *Zp*-221 region is alone sufficient for activation by BCR cross-linking [25].

TLR9 triggering mediates inhibition of *BZLF1* expression in full-length EBV, but does not affect activity of *Zp* reporter constructs

We investigated whether TLR9 triggering affects BCR cross-linking-induced luciferase expression in Akata BL cells transfected with the plasmids pHEBo-221 *Zp* luciferase, pHEBo-554 *Zp* luciferase, pHEBo-900 *Zp* luciferase or pHEBo-2.3Kb *Zp* luciferase. Therefore, we triggered TLR9 using CpG2006, and 2 hours later the BCR was cross-linked with anti-IgG to induce lytic EBV reactivation. Most remarkably, TLR9 triggering in Akata BL cells did not lead to a significant suppression of the luciferase expression from the reporter plasmids after 6 hours (Figure 2) or 16 hours (data not shown). We obtained similar results in transient transfection experiments (data not shown). Thus, TLR9 triggering was not able to suppress BCR-induced expression of *Zp*-controlled luciferase expression in reporter plasmids in Akata BL cells.

The results above stand in contrast to the TLR9 triggering-induced suppression of *BZLF1* expression from genomic EBV. Cross-linking of the BCR in transfected Akata BL cells with anti-IgG for 6 hours resulted in an up-regulation of *BZLF1* mRNA expression and thus in lytic reactivation of the full-length EBV genome. The prior treatment with CpG2006 significantly suppressed BCR-induced *BZLF1* expression levels in transfected and non-transfected Akata BL cells (Figure 3). Thus, TLR9 triggering modulates full-length EBV genome *Zp*-controlled *BZLF1* expression, but does not affect the stably transfected *Zp*-controlled luciferase gene expression.

Chromatin structure and modification differs between full-length EBV genome and transfected reporter plasmids

To investigate further the differential regulation of *Zp* activation in the full-length genomic EBV versus the transfected reporter constructs, we looked at the possible involvement of histone modifications. The histones bound to the promoter region of *BZLF1* were reported to be acetylated and phosphorylated upon BCR cross-linking; an important step in the induction of the lytic cycle of EBV [7, 26-28]. To test whether the discrepant regulation of the full-length EBV genome and the stably transfected reporter constructs might be due to a distinct chromatin structure, we performed ChIP assay on *Zp* reporter constructs.

As others, we observed histone modifications on the *BZLF1* promoter of the EBV viral episome upon BCR cross-linking [7, 26-28]. Similarly to the genomic full length *Zp* promoter, an increased acetylation of histones H3 on the lysine 9 (H3K9ac) could be measured on the pHEBo-221 *Zp* luciferase reporter constructs upon BCR cross-linking (**Figure 4)**. H3K9ac on the genomic *Zp* promoter is inhibited by the TLR9 signaling. On the contrary the activation of TLR9 did not affect the acetylation state of the luciferase reporter constructs. These results show that full-length EBV genome and *Zp* reporter constructs have distinct histones modification and chromatin structures. TLR9 signaling does modify histones on the episomal *Zp* promoter leading to a suppression of *BZLF1* expression, but TLR9 signaling does not affect *Zp*-luciferase expression due to the inability of TLR9 signaling to induce histone modifications on transfected reporter constructs. We conclude that the activity of *Zp* is differentially controlled in the full-length EBV genome versus transfected reporter constructs.

In this study, we aimed to further elucidate the mechanism leading to TLR9 triggering-induced suppression of EBV lytic reactivation in BL cells and examined the effect of TLR9 triggering on the regulation *BZLF1* promoter using reporter constructs of increasing

length, starting with the minimal *Zp*-221. First, we established Akata cells stably transfected with different episomal *Zp* reporter constructs and confirmed activation of the minimal *BZLF1* promoter *Zp*-221 by BCR cross-linking. Next, we showed that TLR9 triggering in Akata BL cells exhibits no effect on the activity of the *Zp*-221-Luc promoter upon BCR cross-linking, nor on larger *Zp* fragments up to 2.3 Kbp, whereas it suppresses expression of *Zp*-controlled *BZLF1* lytic gene in the full-length genomic EBV context. Finally, we showed that TLR9 triggering does not lead to modification of acetylation on histone H3K9 on the *Zp*-221-Luc promoter in contrast to what was observed for *Zp* on genomic EBV. Our results have implications on treatment modalities for EBV-associated cancers based on lytic reactivation of EBV.

The extent of activation of the different *Zp* reporter constructs measured here upon BCR cross-linking was rather similar. Our results are in line with those of Amon et al. [29] who originally engineered the *Zp*-luciferase reporter constructs. One important feature of these reporter constructs is that they are maintained in the nucleus as episomes, since they carry EBV OriP and EBNA1 expression cassette, and allow the generation of cell lines stably carrying the reporter plasmids as episomes. The robustness of these systems was confirmed by the fact that we consistently observed activation of the promoter elements upon BCR cross-linking and that the minimal *Zp*-221 promoter was sufficient to drive expression of the reporter gene. Therefore, these systems should allow mirroring faithfully the regulation of genomic EBV *Zp*.

To our surprise, TLR9 triggering did not suppress luciferase gene expression driven by the *Zp* promoters of different length in reporter plasmids upon BCR cross-linking of Akata BL cells. In contrast, in the very same cells, transcription of *BZLF1* upon BCR cross-linking was suppressed by TLR9 activation. Thus, there must be a fundamental difference in the regulation of *Zp* activity in the genomic context vs. reporter constructs, specifically in the impact that TLR9 signaling has on transcription driven by *Zp* promoter. We have previously

shown that modification of histones on *Zp* promoter is directly correlated with TLR9 induced suppression of *BZLF1* transcription [14]. Others have shown that a plasmid similar to the longest *Zp*-reporter construct (2.3 kbp) used here is embedded into nucleosomal structure [7], supporting the hypothesis that these reporter constructs may faithfully reproduce the wild type nucleosomal structure and regulation. Nevertheless, our functional studies suggest that a difference in the regulation of chromatin reorganization might subsist between *Zp* in full lenght EBV and in recombinant reporter constructs.

As shown here, and earlier by us [14], acetylation of histones H3 and H4, as well as phosphorylation of H3, are induced by BCR cross-linking on EBV genomic *Zp* in Akata BL cells, and these modifications are suppressed by TLR9 signaling. Thus, we analyzed the acetylation status of histone H3K9 in *Zp* reporter constructs. This histone modification was also induced by BCR cross-linking in the *Zp* reporter constructs analyzed. On the other hand, TLR9-induced inhibition of acetylation on histone H3K9 observed for the genomic *Zp* did not take place on the *Zp* reporter constructs, indicating that TLR9 triggering has a different effect on chromatin structure associated with genomic EBV than that associated with reporter plasmids. This, in turn, strongly suggests that the suppressive effect of TLR9 signaling on *Zp* promoter activation is linked to a specific chromatin conformation associated with genomic EBV.

Conclusion

Our findings further support the importance of chromatin reorganization in the regulation of transcription of *BZLF1* (Figure 5) and therefore in the balance between EBV latency and lytic reactivation in cancer cells, as observed by the efficient induction of lytic EBV by a specific set of histone deacetylase (HDAC) inhibitors [30]. Notably, histone methylation has been shown to be associated with latent EBV *BZLF1* promoter region and to be at least partly involved in the maintenance of EBV latency in Raji BL cells [31]. Further elucidation of

chromatin reorganization in the regulation of transcription of *BZLF1* is important in view of the fact that lytic EBV reactivation is being explored as a specific cancer cell-targeted therapy for EBV-associated malignancies [5] and improved HDAC inhibitors are being developed for the treatment of diverse malignancies. An enhanced understanding of the regulation of EBV lytic reactivation by cellular signaling pathways, including TLR9 signaling, will allow optimizing therapeutic strategies based on manipulation of chromatin remodeling.

Methods

Cell culture

EBV-positive Akata BL cells were cultured in RPMI 1640 medium (BioConcept, Allschwil, Switzerland) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), and 10 % heat-inactivated fetal calf serum. Transfected cells were selected with 0.3 mg/ml hygromycin B (Invitrogen, California, USA). The human epithelial cell line HeLa was cultured in DMEM medium (BioConcept) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), and 10 % heat-inactivated fetal calf serum. Both cell lines were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Plasmids

Akata BL cells were transfected with a pHEBo vector containing different Zta promoter (*Zp*) regions followed by a gene encoding the firefly luciferase. The pHEBo luciferase, the pHEBo-221 *Zp* luciferase, and the pHEBo-554 *Zp* luciferase plasmids were kindly provided by P.J. Farrell [32]. The pHEBo-900 *Zp* luciferase and pHEBo-2.3 Kb *Zp* luciferase plasmids were cloned in our laboratory (see below).

Cloning of pHEBo-900 *Zp* luciferase and pHEBo-2.3 Kb *Zp* luciferase

Total DNA from Akata BL cells was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. The -900 bp and -2.3 Kb *Zp* regions were amplified from Akata BL cells by PCR using the following primers:

- 900 bp *Zp*:

Forward: 5'-GCT AGC GCC CAA CCG TGG TCA AAC TC-3'

Reverse: 5'-CTC GAG GTG CAA TGT TTA GTG AGT TAC-3'.

- 2.3 Kb *Zp*:

Forward: 5'-GTC GAC TCC TTG GGG GAG TAG TAG CTT-3'

Reverse: 5'-GCT AGC GTG CAA TGT TTA GTG AGT TAC -3'.

The PCR amplifications were cloned into the pCR 2.1-TOPO vector and amplified using TOP10 competent cells according to supplier's protocol (TOPO TA Cloning Kit, Invitrogen). The TOPO vector containing the -900 *Zp* was digested with *Bcu*1 and *Xho*1 (New England Biolabs, Ipswich, MA, USA) and the -2.3 Kb *Zp* with *Nhe*1 and *Sal*1 (New England Biolabs). The minimal promoter *Zp*-221 from the pHEBo-221 *Zp* luciferase was cut out using *Nhe*1 and *Xho*1. To avoid self-ligation, the vector was incubated for 1 hour with calf intestine alkaline phosphatase (CIP). Purification was performed with the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol.

Ligation was realized in two ratios. 1:3 (insert: vector) with 150 ng vector and 1:5 with 100 ng vector. Vector and insert were mixed with 2.5 µl 10x Buffer for T4 DNA Ligase (New England Biolabs), 1 µl T4 DNA Ligase (New England Biolabs) and water to a total of 25 µl. Samples were incubated overnight at 16°C.

Amplification of ligated plasmids was performed in One Shot Stbl3 chemically competent *Escherichia coli* according to manufacturer's protocol (Invitrogen, Basel, Switzerland), followed by plasmid isolation using the GeneElute HP Plasmid Midiprep Kit (Sigma-Aldrich, St. Louis, MO, USA). Plasmids were sequenced through the *Zp* region to confirm their identity. Sequencing was performed at Microsynth (Balgach, St. Gallen, Switzerland). The following primers were used: 5'-CTT TAT GTT TTT GGC GTC TTC CA-3' (GLprimer2; for the -221 *Zp*, -554 *Zp*, -900 *Zp* and the 2.3 Kb *Zp*); 5'-CGG CTT TGG CAG GAA CAT AC-3' (only for the -900 bp *Zp*); and 5'-AGA GCC CTG ACA TCC TTA AC-3' (only for the 2.3 Kb *Zp*).

Transfection

Nucleoporation of Burkitt's lymphoma cells

Harvested cells were pelleted and resuspended in Cell Line Nucleofecter Solution T (Lonza Group Ltd, Basel, Switzerland) to a final concentration of 3.5 x 10^6 cells / 1000 µl, followed by addition of 7 µg of the appropriate plasmid. The mixture was transferred to an Amaxa cuvette. Program A-23 was run by an amaxa Nucleofecter II (Amaxa, since 2008 Lonza Group Ltd, Basel, Switzerland). Pre-warmed culture medium (500 µl) was added to the cuvette and cells were transferred immediately to 6.5 ml pre-warmed RPMI 1640 in a culture flask. The cuvette was washed several times with culture medium to make sure that the remaining cells were also transferred to the flask. Transfection efficiency was determined 24 to 72 h after transfection by a sample nucleoporated with 4 µg of pmaxGFP (Lonza), using a Leica DMIL microscope (Leica Microsystems, Heerbrugg, Switzerland).

$CaCl_2$ transfection of HeLa cells

HeLa cells (1.5 x 10^5 cells / ml in complete DMEM) were seeded on a 6-well tissue culture plate (250,000 cells per well). The next day, transfection was performed by diluting the according amounts of plasmids with HEPES-buffered water (2.5 mM HEPES in water) to a total volume of 35 µl. Pre warmed (37 °C) 0.5 M $CaCl_2$ (35 µl) was added. While vortexing, the mixture was pipetted to 70 µl HEBS 2x (37°C) and then incubated at RT for 20 to 30 minutes. Cells were added and incubated for one day at 37°C.

Cell treatment

Cells were treated with the histone deacetylase inhibitor Trichostatin A (TSA; Sigma) (50 ng/ml final concentration). At 12 h to 14 h later, ODN CpG-2006 (Eurogentec, Seraing, Belgium) with a final concentration of 0.5 µM was added. Two hours polyclonal rabbit anti-

human IgG (100 µg / ml) (Dako Denmark, Glostrup, Denmark) was added to induce lytic EBV infection. Cells were harvested 4, 6 and 16 h after inducing lytic EBV infection.

Luciferase Assay

The harvested cells were resuspended in 150 µl Reporter Lysis Buffer 5x (Promega, Madison, WI, USA) and incubated for 15 minutes at 37°C gentle rocking. To guarantee perfect lysis, cells were frozen on dry ice and then unfrozen at 37°C. Tubes were vortexed for 15 seconds and centrifuged for 2 minutes at 16,000 g and 4°C. The supernatant was transferred to a new tube. Triplicates of 20 µl cell extract per well were pipetted on a flat-bottom 96-well plate. 100 µl Assay Substrate (Promega) per well were added. Luminescence was measured with a sensitivity of 250 nm by the Synergy HT reader (Bio-Tek, Winoosiki, VT, USA).

RNA isolation and quantitative Real time PCR (TaqMan)

Cells were harvested 4, 6 and 16 h after the induction of lytic EBV infection by anti-IgG. RNA was isolated using the Qiagen RNEasy Mini Kit (Qiagen) according to the manufacturer's protocol. DNAse treatment (DNAfree; Ambion Europe, Huntington, Cambridgeshire, UK) was performed, and RNA concentration was measured using NanoDrop (ND-1000 spectrophotometer). An amount of 1 µg of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, United Kingdom) as recommended by the supplier. Primer (300nM), probe (200nM), TaqMan master mix, cDNA and DEPEC-treated water were pipetted into a 376-well plate. Quantitative real time PCR (qRT-PCR) was performed with validated TaqMan systems for the housekeeping gene *hydroxymethylbilane synthase* (*HMBS*) and the EBV immediate-early lytic gene *BZLF1* [13, 33]. Primer and probes sequences: *HMBS*-forward 5`-GCTCGCATACAGACGGACAG-3`, *HMBS*-reverse 5`-AGGCCAGGGTACGAGGCTT-3`, *HMBS*-probe 5`-TGGTGGCAACATTG-3`; *BZLF1*-forward 5`-CACGACGRACAAGGAAAC-3`, *BZLF1*-reverse 5`-CGCTTTATTTCTAGTTCAGAATCGC-3`, *BZLF1*-probe 5`-

CAGCCAGAATCGCTGGAGGAA-3`. 18S rRNA primer and probes used were from Applied Biosystems.

The 7900 HT Fast Real Time PCR System (Applied Biosystems) with the appropriate temperature profile was used as follows. Initial denaturation for 10 min at 95°C, 40 cycles with denaturation for 15 seconds at 95°C and elongation for 1 minute at 60°C. Threshold cycle (Ct) numbers were determined using the SDS 2.2 software (Applied Biosystems). Gene transcription was normalized to mRNA expression of *HMBS* or 18S rRNA by calculating Ct(housekeeping gene)-Ct(*BZLF1*) resulting in threshold cycle (ΔCt) values. Results were analyzed calculating $2^{\Delta Ct}$.

Chromatin immunoprecipitation (ChIP) analysis

ChIP was preformed according to the manufacturer's instructions (ChIP-IT Express, Active Motif, La Hulpe, Belgium). Briefly, 1.5 x 10^7 transfected cells were fixed by formaldehyde. Cells were lysed and homogenized using a 25G syringe. Chromatin shearing was performed by 15 x 20 seconds sonication at 25% with a 30 seconds break between each pulse using the Epishear™ Probe Sonicator (Active Motif, La Hulpe, Belgium). 7-25 µg (40 µl) sheared chromatin was incubated over night on a rotating wheel with 3 µg H3K9Ac antibody (AbCam, Cambridge, UK) and protein G magnetic beads. Washing steps and elution of chromatin was followed by proteinase treatment. The DNA purification was performed according to the QIAquick® PCR Purification Kit (Qiagen, Hombrechtikon, Switzerland). Quantitative real time PCR (qRT-PCR) was performed for the antibody selected sample as well as their corresponding 25% input samples with the following primers: Genomic *Zp*: ZP4 F 5'-GAGCCACAGGCATTGCTAA-3', Rev BZ1 967 5'AAGATAGCAAAGGTGGCCGG-3', Reporter *Zp*: ZP4 F 5'-GAGCCACAGGCATTGCTAA-3', Rev LUC-5178 5'-CAGTACCGGAATGCCAAGCT-3', both pairs with the probe: 5'-AAGCCAAGGCACCAGCCTCC-3'.

The 7900 HT Fast Real Time PCR System (Applied Biosystems) with the appropriate temperature profile was used as follows. Initial denaturation for 10 min at 95°C, 40 cycles with denaturation for 15 seconds at 95°C and elongation for 1 minute at 60°C. Threshold cycle (Ct) numbers were determined using the SDS 2.2 software (Applied Biosystems). The IP efficiency was calculated using the equation: $25\% \times 2^{(Ct\ 25\%\ input\ sample - Ct\ IP\ sample)}$.

Statistical Analysis

Values are reported as means (± standard error of the mean) from at least 3 independent biological replicates. Statistical analysis to determine the corresponding P value was performed using ANOVA (Bonferroni post-test) or unpaired t-tests. We computed all statistics with Prism 4 (Graph Pad, San Diego, CA, USA). P values < 0.05 were regarded as statistically significant.

List of abbreviations

BCR: B-cell receptor; BL: Burkitt's lymphoma; CpG: cytosine-phosphatidyl-guanosine; EBV: Epstein-Barr virus; HDAC: histone deacetylase; ODN: oligodeoxynucleotide; PCR: polymerase chain reaction; RLU: relative luciferase units

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LZ, MW, and MJ participated in the design of the study, performed experiments and wrote the initial draft of the manuscript. NAW performed experiments. JAS cloned pHEBo-900 *Zp* luciferase and pHEBo-2.3 Kb *Zp* luciferase. CB participated in the design of the study. MB and DN conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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References

1. Wen W, Iwakiri D, Yamamoto K, Maruo S, Kanda T, Takada K: **Epstein-Barr virus BZLF1 gene, a switch from latency to lytic infection, is expressed as an immediate-early gene after primary infection of B lymphocytes.** *J Virol* 2007, **81:**1037-1042.
2. Rowe DT: **Epstein-Barr virus immortalization and latency.** *Front Biosci* 1999, **4:**D346-371.
3. Epstein MA, Achong BG, Barr YM: **Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma.** *Lancet* 1964, **1:**702-703.
4. Rochford R, Cannon MJ, Moormann AM: **Endemic Burkitt's lymphoma: a polymicrobial disease?** *Nat Rev Microbiol* 2005, **3:**182-187.
5. Amon W, Farrell PJ: **Reactivation of Epstein-Barr virus from latency.** *Rev Med Virol* 2005, **15:**149-156.
6. Iwakiri D, Takada K: **Phosphatidylinositol 3-kinase is a determinant of responsiveness to B cell antigen receptor-mediated Epstein-Barr virus activation.** *J Immunol* 2004, **172:**1561-1566.
7. Jenkins PJ, Binne UK, Farrell PJ: **Histone acetylation and reactivation of Epstein-Barr virus from latency.** *J Virol* 2000, **74:**710-720.
8. Rowe M, Rowe DT, Gregory CD, Young LS, Farrell PJ, Rupani H, Rickinson AB: **Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells.** *Embo J* 1987, **6:**2743-2751.
9. Flemington E, Speck SH: **Autoregulation of Epstein-Barr virus putative lytic switch gene BZLF1.** *J Virol* 1990, **64:**1227-1232.

10. di Renzo L, Altiok A, Klein G, Klein E: **Endogenous TGF-beta contributes to the induction of the EBV lytic cycle in two Burkitt lymphoma cell lines.** *Int J Cancer* 1994, **57:**914-919.

11. Takada K: **Cross-linking of cell surface immunoglobulins induces Epstein-Barr virus in Burkitt lymphoma lines.** *Int J Cancer* 1984, **33:**27-32.

12. Takada K, Ono Y: **Synchronous and sequential activation of latently infected Epstein-Barr virus genomes.** *J Virol* 1989, **63:**445-449.

13. Ladell K, Dorner M, Zauner L, Berger C, Zucol F, Bernasconi M, Niggli FK, Speck RF, Nadal D: **Immune activation suppresses initiation of lytic Epstein-Barr virus infection.** *Cell Microbiol* 2007, **9:**2055-2069.

14. Zauner L, Melroe GT, Sigrist JA, Rechsteiner MP, Dorner M, Arnold M, Berger C, Bernasconi M, Schaefer BW, Speck RF, Nadal D: **TLR9 triggering in Burkitt's lymphoma cell lines suppresses the EBV BZLF1 transcription via histone modification.** *Oncogene* 2010, **29:**4588-4598.

15. Akira S, Takeda K, Kaisho T: **Toll-like receptors: critical proteins linking innate and acquired immunity.** *Nat Immunol* 2001, **2:**675-680.

16. Dorner M, Brandt S, Tinguely M, Zucol F, Bourquin JP, Zauner L, Berger C, Bernasconi M, Speck RF, Nadal D: **Plasma cell toll-like receptor (TLR) expression differs from that of B cells, and plasma cell TLR triggering enhances immunoglobulin production.** *Immunology* 2009, **128:**573-579.

17. Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S: **Host innate immune receptors and beyond: making sense of microbial infections.** *Cell Host Microbe* 2008, **3:**352-363.

18. Coban C, Igari Y, Yagi M, Reimer T, Koyama S, Aoshi T, Ohata K, Tsukui T, Takeshita F, Sakurai K, et al: **Immunogenicity of whole-parasite vaccines against**

Plasmodium falciparum involves malarial hemozoin and host TLR9. *Cell Host Microbe* 2010, **7:**50-61.

19. Takeshita F, Gursel I, Ishii KJ, Suzuki K, Gursel M, Klinman DM: **Signal transduction pathways mediated by the interaction of CpG DNA with Toll-like receptor 9.** *Semin Immunol* 2004, **16:**17-22.

20. Manet E, Gruffat H, Trescol-Biemont MC, Moreno N, Chambard P, Giot JF, Sergeant A: **Epstein-Barr virus bicistronic mRNAs generated by facultative splicing code for two transcriptional trans-activators.** *Embo J* 1989, **8:**1819-1826.

21. Speck SH, Chatila T, Flemington E: **Reactivation of Epstein-Barr virus: regulation and function of the BZLF1 gene.** *Trends Microbiol* 1997, **5:**399-405.

22. Montalvo EA, Cottam M, Hill S, Wang YJ: **YY1 binds to and regulates cis-acting negative elements in the Epstein-Barr virus BZLF1 promoter.** *J Virol* 1995, **69:**4158-4165.

23. Montalvo EA, Shi Y, Shenk TE, Levine AJ: **Negative regulation of the BZLF1 promoter of Epstein-Barr virus.** *J Virol* 1991, **65:**3647-3655.

24. Speck SH, Chatila T, Flemington E: **Reactivation of Epstein-Barr virus: regulation and function of the BZLF1 gene.** *Trends Microbiol* 1997, **5:**399-405.

25. Binne UK, Amon W, Farrell PJ: **Promoter sequences required for reactivation of Epstein-Barr virus from latency.** *J Virol* 2002, **76:**10282-10289.

26. Countryman JK, Gradoville L, Miller G: **Histone hyperacetylation occurs on promoters of lytic cycle regulatory genes in Epstein-Barr virus-infected cell lines which are refractory to disruption of latency by histone deacetylase inhibitors.** *J Virol* 2008, **82:**4706-4719.

27. Gruffat H, Manet E, Sergeant A: **MEF2-mediated recruitment of class II HDAC at the EBV immediate early gene BZLF1 links latency and chromatin remodeling.** *EMBO Rep* 2002, **3:**141-146.

28. Ramasubramanyan S, Osborn K, Flower K, Sinclair AJ: **Dynamic chromatin environment of key lytic cycle regulatory regions of the Epstein-Barr virus genome.** *J Virol* 2012, **86:**1809-1819.

29. Amon W, Binne UK, Bryant H, Jenkins PJ, Karstegl CE, Farrell PJ: **Lytic cycle gene regulation of Epstein-Barr virus.** *J Virol* 2004, **78:**13460-13469.

30. Ghosh SK, Perrine SP, Williams RM, Faller DV: **Histone deacetylase inhibitors are potent inducers of gene expression in latent EBV and sensitize lymphoma cells to nucleoside antiviral agents.** *Blood* 2012, **119:**1008-1017.

31. Murata T, Kondo Y, Sugimoto A, Kawashima D, Saito S, Isomura H, Kanda T, Tsurumi T: **Epigenetic Histone Modification of Epstein-Barr Virus BZLF1 Promoter during Latency and Reactivation in Raji Cells.** *J Virol* 2012, **86:**4752-4761.

32. Sugden B, Marsh K, Yates J: **A vector that replicates as a plasmid and can be efficiently selected in B-lymphoblasts transformed by Epstein-Barr virus.** *Mol Cell Biol* 1985, **5:**410-413.

33. Rechsteiner MP, Berger C, Zauner L, Sigrist JA, Weber M, Longnecker R, Bernasconi M, Nadal D: **Latent membrane protein 2B regulates susceptibility to induction of lytic Epstein-Barr virus infection.** *J Virol* 2008, **82:**1739-1747.

Figure legends

Figure 1: BCR cross-linking induces expression of luciferase in Akata cells stably transfected with different *BZLF1* reporter constructs. Akata cells stably transfected with the plasmids pHEBo luciferase, pHEBo-221 *Zp* luciferase, pHEBo-554 *Zp* luciferase, pHEBo-900 *Zp* luciferase, and transiently transfected with pHEBo-2.3 Kb *Zp* luciferase were treated with anti-IgG for 6 hours. The activation of the *BZLF1* promoter regions was measured by luciferase assay. Experiments were performed three times, with cells originating from one transfection for pHEBo luciferase, pHEBo-221 *Zp* luciferase and pHEBo-554 *Zp* luciferase and originating from three different transfections for pHEBo-2.3Kb *Zp* luciferase. Relative luciferase units (RLUs) were calculated by dividing the absolute luciferase values of anti-IgG treated cells by the absolute luciferase values of mock treated cells. Values are reported as means +/- standard error of the mean. Abbreviations are as follows: *: P< 0.05, **: P<0.01.

Figure 2: TLR9 triggering by CpG does not significantly affect BCR-induced up-regulation of luciferase expression in Akata cells transfected with pHEBo-221 *Zp* luciferase, pHEBo-554 *Zp* luciferase, pHEBo-900 *Zp* luciferase or pHEBo-2.3Kb Zp luciferase. Activity of the enzyme luciferase after treatment with CpG and anti-IgG is shown for the cells transfected with different Zp promoter constructs. Cells were harvested 6 hours after adding anti-IgG. All experiments were performed three times. Relative luciferase units (RLUs) were calculated by dividing the absolute luciferase values of anti-IgG treated cells by the absolute luciferase values of mock treated cells. Values are reported as means +/- standard error of the mean. Abbreviations are as follows: *: P< 0.05, **: P<0.01.

Figure 3: TLR9 triggering suppresses BCR-induced *BZLF1* mRNA expression. Untransfected and transfected Akata cells were treated with anti-IgG and CpG. Reactivation

of EBV in Akata cells was quantified by measuring *BZLF1* mRNA expression over the housekeeping gene *HMBS*. Cells were harvested 6 hours after adding anti-IgG.

Figure 4: TLR9 signaling influences histone H3 acetylation on lysine 9 on the genomic *Zp* promoter of *BZLF1* but not on stably transfected pHEBo-221 *Zp* luciferase reporter plasmid.

Akata cells stably transfected with pHEBo *Zp*-221 luciferase plasmids were treated with CpG2006, anti-IgG or both. Cells were harvested and ChIP assay was performed with antibodies against H3K9ac. One ChIP using untreated lysed cells was performed with a normal rabbit IgG antibody as negative control and one ChIP was performed with a anti-H3 antibody as positive control. ChIP reactions were followed by a q-rtPCR for the genomic *Zp* promoter region and for the pHEBo *Zp*-221 luciferase region based on the reporter plasmid. Shown in ng is the quantity of isolated chromatin from each ChIP reaction. One representative experiment is shown out of at least three independent experiments.

Figure 5: Regulation of BZLF1 promoter Zp activity on genomic EBV by TLR9 signalling.

Viral lytic reactivation is characterized by histone acetylation on the promoter region of the immediate early lytic gene BZLF1 contributing to its expression. It has been observed that BCR cross-linking on Burkitt‘s lymphoma cells, results in the acetylation of histones on the Zp promoter and to the expression of Zta. It is still unclear if BCR cross-linking leads to histone acetylation through direct (or indirect) activation of histone acetyltransferases (HAT) and / or through inhibition of histone deacetylases (HDAC). We showed that TLR9 activation hinders BCR cross-linking’s effect on Zp histones and thus to abolish BZLF1 expression. We hypothesise that this repression affects the balance between HAT and HDAC activity either

by activating HDACs or by inhibiting HATs. As result, the histones on the Zp promoter are deacetylated when TLR9 is triggered prior to viral lytic reactivation.

Figure 1

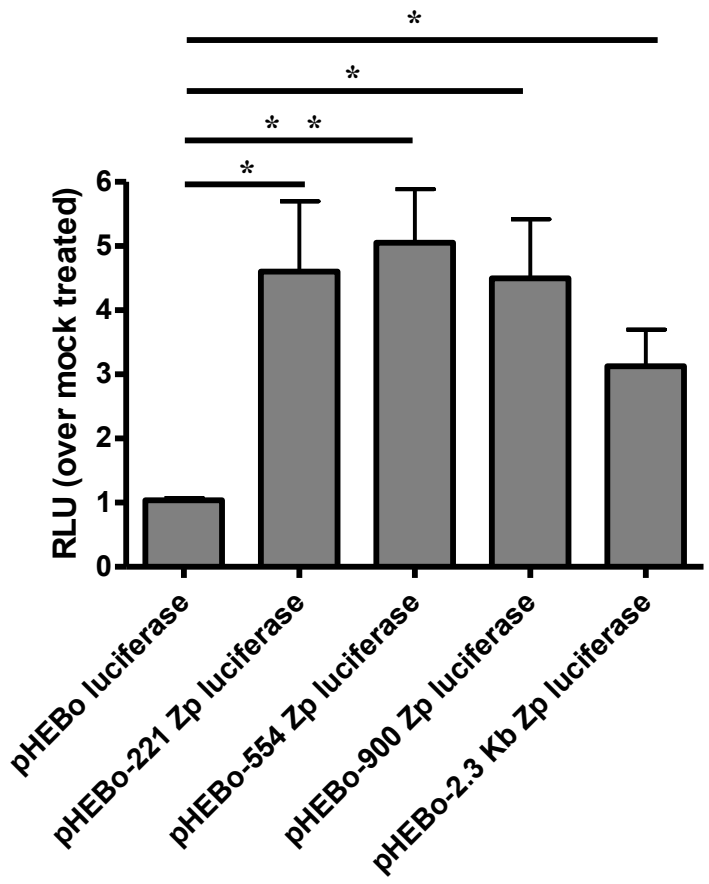

*
*
* *
*
RLU (over mock treated)
0
1
2
3
4
5
6
pHEBo luciferase
pHEBo-221 Zp luciferase
pHEBo-554 Zp luciferase
pHEBo-900 Zp luciferase
pHEBo-2.3 Kb Zp luciferase

Figure 2:

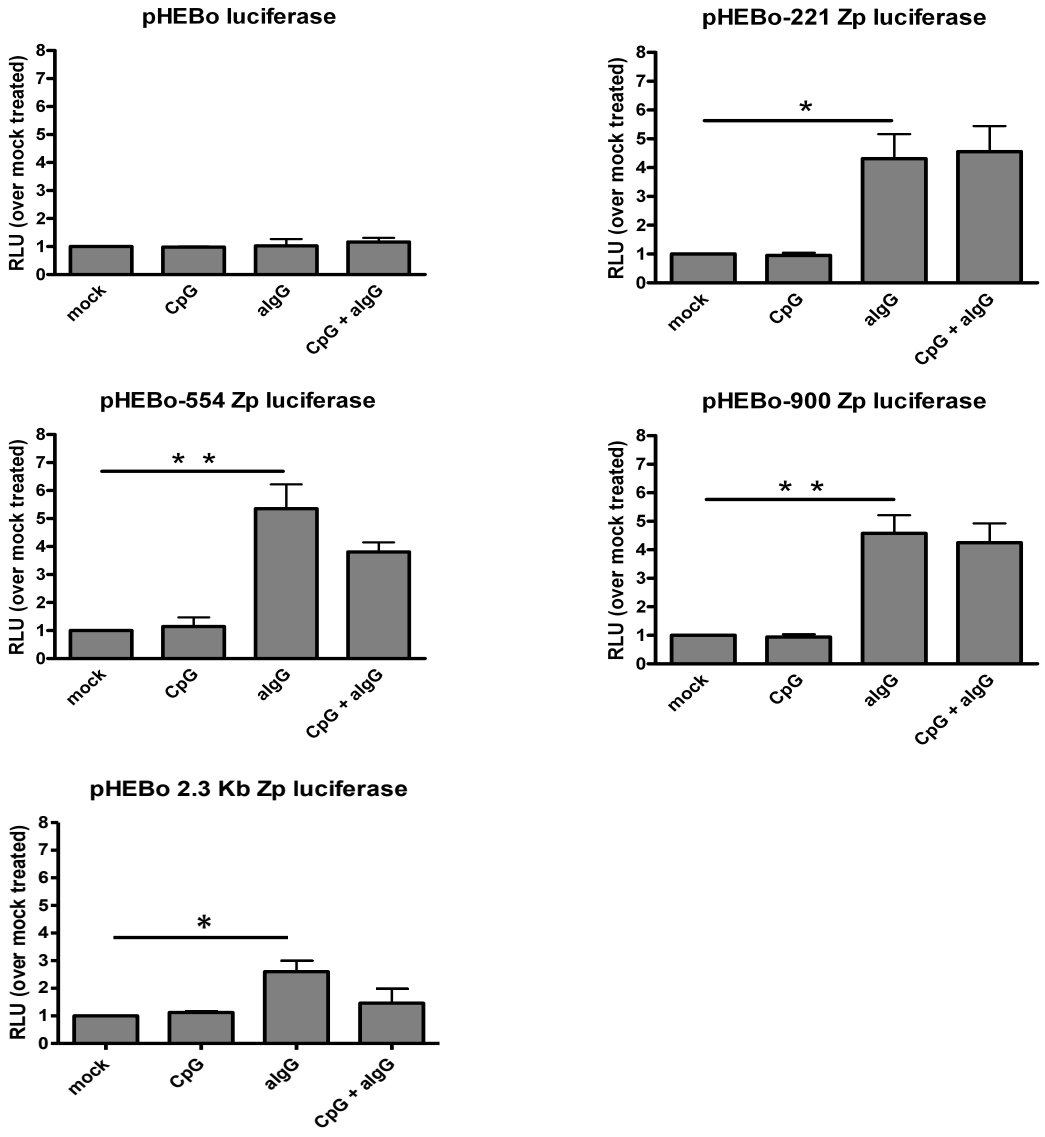
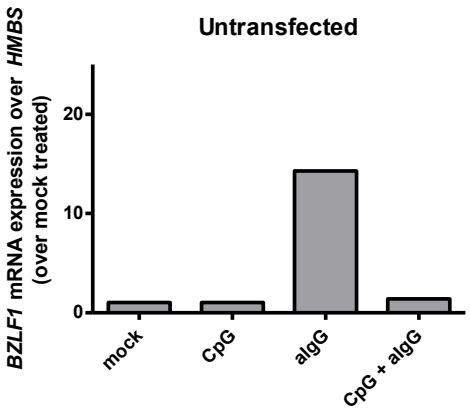
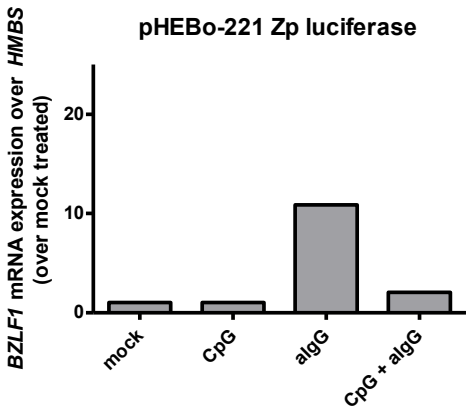
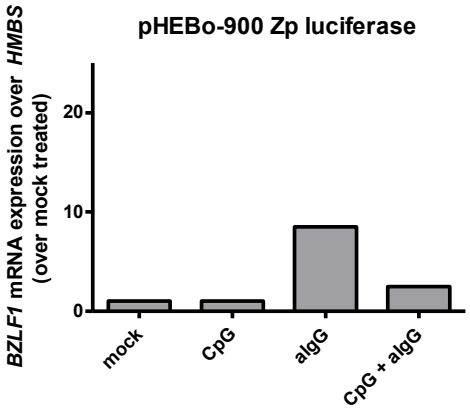
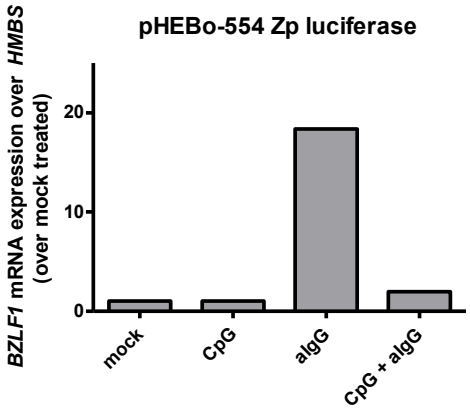

pHEBo luciferase
RLU (over mock treated)
mock
CpG
algG
CpG + algG
pHEBo-221 Zp luciferase
*
pHEBo-554 Zp luciferase
* *
pHEBo-900 Zp luciferase
* *
pHEBo 2.3 Kb Zp luciferase
*

Figure 3:


Untransfected
BZLF1 mRNA expression over HMBS (over mock treated)
0
10
20
mock
CpG
algG
CpG + algG


pHEBo-221 Zp luciferase
BZLF1 mRNA expression over HMBS (over mock treated)
0
10
20
mock
CpG
algG
CpG + algG


pHEBo-900 Zp luciferase
BZLF1 mRNA expression over HMBS (over mock treated)
0
10
20
mock
CpG
algG
CpG + algG


pHEBo-554 Zp luciferase
BZLF1 mRNA expression over HMBS (over mock treated)
0
10
20
mock
CpG
algG
CpG + algG

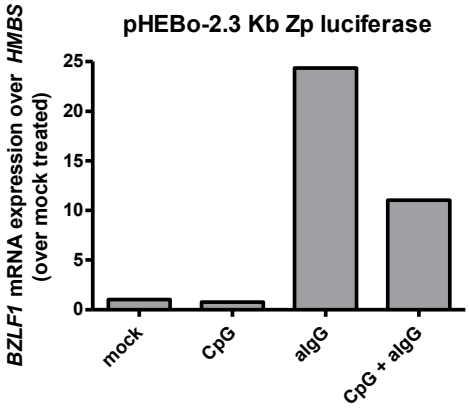
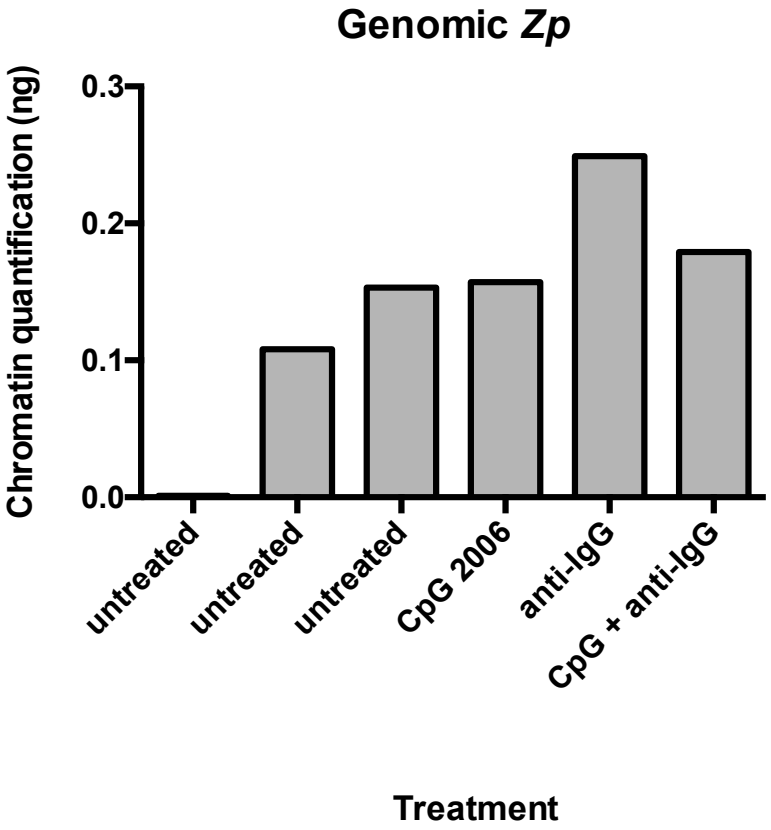

pHEBo-2.3 Kb Zp luciferase
BZLF1 mRNA expression over HMBS (over mock treated)
0
5
10
15
20
25
mock
CpG
algG
CpG + algG

Figure 4


Genomic Zp
Chromatin quantification (ng)
0.3
0.2
0.1
0.0
untreated
untreated
untreated
CpG 2006
anti-IgG
CpG + anti-IgG
Treatment

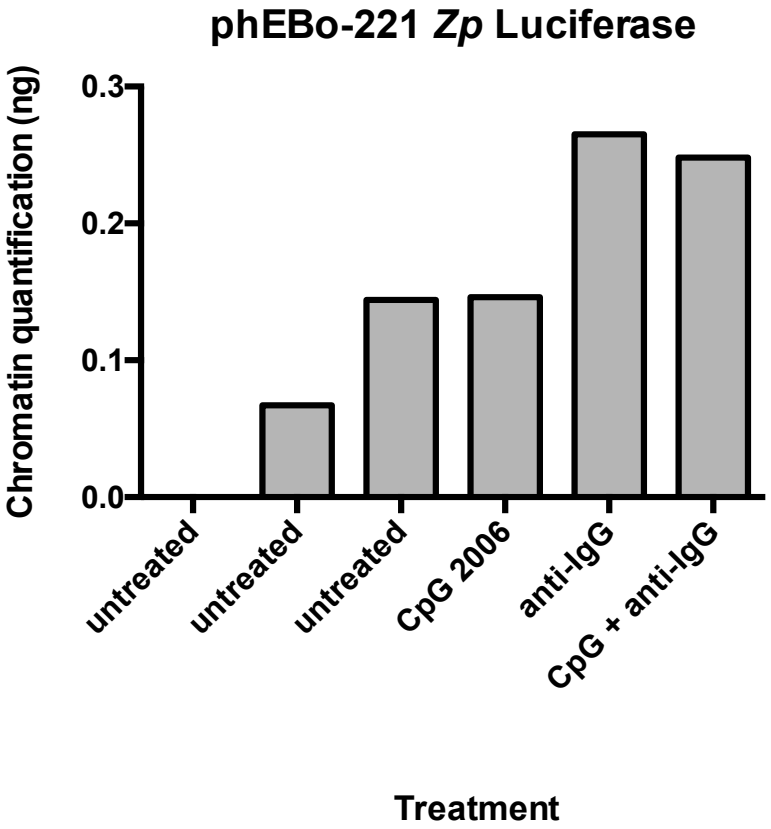
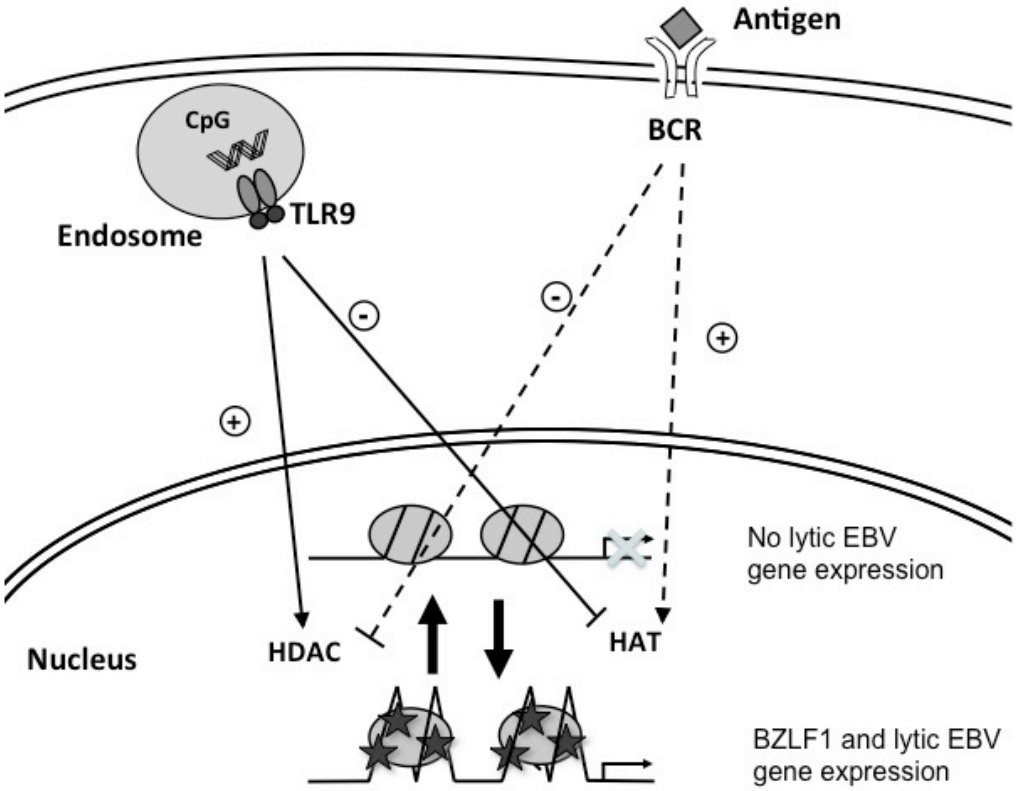
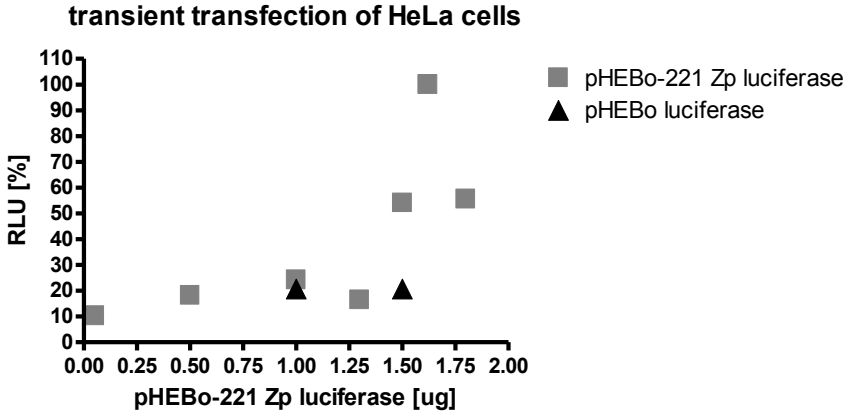

phEBo-221 Zp Luciferase
Chromatin quantification (ng)
0.3
0.2
0.1
0.0
untreated
untreated
untreated
CpG 2006
anti-IgG
CpG + anti-IgG
Treatment

Figure 5:


Antigen
BCR
CpG
TLR9
Endosome
No lytic EBV
gene expression
Nucleus
HDAC
HAT
BZLF1 and lytic EBV
gene expression

Supplementary Figure 1: Functionality of *BZLF1* reporter constructs. Seven different concentrations of pHEBo-221 *Zp* luciferase plasmid were co-transfected by $CaCl_2$ transfection with a constant amount of pLenti4-*BZLF1* plasmids. Co-transfection with pHEBo luciferase and *BZLF1* pLenti4 served as a control. Relative luciferase units (RLUs) were calculated by dividing the absolute luciferase values by the appropriate beta-galactosidase values, and setting of the highest value to 100 %.



Manuscript III:

TLR9 agonists induced cell death in Burkitt's lymphoma cells is variable and influenced by *TLR9* polymorphism

Running title: Burkitt's lymphoma and *TLR9* polymorphisms

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Abstract

Toll-like receptor 9 (TLR9) triggering is a promising novel strategy to combat cancer as it induces innate and adaptive immunity responses. B-cell lymphoma is unique in this context as tumor cells express TLR9 and may harbor latent Epstein-Barr virus (EBV), a gammaherpesvirus with remarkable oncogenic potential when latent. Latent EBV may be promoted by TLR9 triggering via suppression of lytic EBV. Here, we elaborated an initial assessment of the impact of TLR9 triggering on EBV-positive and EBV-negative B-cell lymphoma using Burkitt's lymphoma (BL) cell lines as an *in vitro* model. We show that, independent of the presence of EBV, the TLR9 ligand ODN CpG-2006 may or may not induce caspase-dependent cell death in BL cells. Moreover, ODN CpG-2006-induced cell death responses of BL cells were associated with *TLR9* single nucleotide polymorphisms (SNPs) rs5743836 or rs352140, which we detected in primary BL tumors and in peripheral blood from healthy individuals at similar frequencies. Thus, our findings suggest that the effect of TLR9 agonists on BL cells should be tested *in vitro* prior to installment of therapy and *TLR9* SNPs in BL patients should be determined as potential biological markers for the therapeutic response to treatment targeting innate immunity.

Keywords: Burkitt's lymphoma, Epstein-Barr virus, Toll-like receptor TLR 9 agonists, polymorphism, CpG

Abbreviations

BL	Burkitt's lymphoma
Ct	Cycle threshold
EBV	Epstein-Barr virus
hIL-10	human interleukin 10
MFI	mean fluorescence intensity
MyD88	myeloid differentiation factor D 88
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
ODN	oligodeoxynucleotide
PBS	phosphate-buffered saline
qRT-PCR	quantitative real-time polymerase chain reaction
rhIL-10	recombinant human interleukin 10
TLR	Toll-like receptor

Introduction

Toll-like receptors (TLRs) are important players of the innate immune system [1], and expression of the ten TLRs known in humans [2, 3] depends on the cell subset and differentiation status [4-6]. TLR9 is preferentially expressed by B-cells and plasmacytoid dendritic cells (reviewed in ref. [1]). Synthetic TLR9 ligands, short oligodeoxynucleotides (ODN) with unmethylated CpG motifs [7], activate TLR9 that recruits the adaptor protein myeloid differentiation factor 88 (MyD88) and induces a cascade leading to the nuclear translocation of the transcription factor nuclear factor κB (NF-κB) [2].

The immune response following TLR9 engagement is highly desirable for cancer treatment [8]. Synthetic TLR9 agonists are regarded as potential anti-cancer agents. Clinical trials have shown promising results for the treatment of various cancer types with CpG ODNs [9]. Their effects can be indirect by enhancing the anti-tumor immune response or direct by inducing apoptosis in the malignant cells. Direct effects would be expected for B-cell malignancies as they express TLR9. B-cell activation can eventually lead to activation-induced cell death of cancer cells and therefore support anti-cancer treatment [10]. Nevertheless, the TLR9 agonist effects vary strongly between B-cell cancer types, and the responses of B-cell lymphomas to ODN CpG-2006 show high variability [11]. Thus, the effects of CpG ODNs on B-cell malignancies are not predictable. Moreover, CpG ODN treatment has tumor-promoting effects on benign B-cells and strongly enhances their proliferation and differentiation [12-14].

Stimulation of TLR9 with ODN CpG-2006 suppresses lytic reactivation of Epstein-Barr virus (EBV), a B-cell tropic gamma-herpesvirus, and can thereby promote latent EBV [15, 16]. The latter is associated with several types of B-cell lymphomas including Burkitt's lymphoma (BL) [17]. EBV is present in nearly all cases of the high-incidence form of BL ('endemic BL'), in up to 85% of the intermediate-incidence cases, and in 15% of the low-incidence cases ('sporadic' BL). Up to 40% of BL in human immunodeficiency virus carriers

harbor EBV [17]. Since latent EBV exhibits unique growth transformation potential on B-cells *in vitro* [18] and TLR9 triggering enhances EBV-induced B-cell transformation [19] and promotes latent EBV *in vitro* [15, 16], TLR9 stimulation in the treatment of EBV-positive BL could provoke detrimental rather than beneficial outcomes.

Single nucleotide polymorphisms (SNPs) of *TLR9* are associated with increased risk for certain B-cell lymphomas [20] and have the potential to exhibit deregulation of signaling thereby promoting tumorigenesis. The responses of *TLR9* SNPs in B-cell tumors to CpG ODN treatment are not reported.

Here, we used BL cell lines to model direct effects of TLR9 stimulation on malignant cells, investigate the influence of EBV, and assess the impact of *TLR9* SNPs, which we found in primary BL samples or in healthy primary cells.

Results

TLR9 triggering alters gene expression and activates Akata cells in a MyD88-dependent manner. CpG ODNs activate B-cells by impacting on gene expression [21]. We asked how the gene expression pattern of BL cells is affected by TLR9 triggering using CpG ODN. We performed a microarray analysis comparing ODN CpG-2006-treated versus untreated Akata cells. Most of the ≥ 2-fold changes in gene expression were up-regulation and only few down-regulation. Among up-regulated genes, we were mainly interested in those involved in cytokine (*hIL-10*) and chemokine expression (*CXCL10*), B-cell activation (*CD40*), transcription (*NF-κB*) and apoptosis (*FAS*) as these genes are those that preferentially determine cell proliferation and survival (Suppl. Table 1). Thirteen genes including two regulating cell cycle, one transcription factor, one involved in B-cell differentiation and apoptosis, four of diverse functions, and four of unknown function showed down-regulation following ODN CpG-2006 treatment (Suppl. Table 2).

We earlier confirmed that TLR9 triggering of Akata cells with ODN CpG-2006 increases hIL-10 expression and leads to the translocation of NF-κB to the nucleus [16]. To validate a marker for B-cell activation, in this context, we measured CD40 expression by flow cytometry. CD40 expression was increased upon ODN CpG-2006 treatment (Figure 1a). After 48 hours, CD40 expression on ODN CpG-2006-treated cells was increased 2.7-fold compared to untreated cells (Figure 1a and 1b). Surprisingly, the control ODN GpC-2006, which is similar to ODN CpG-2006 but lacks the CpG motifs, also led to a 2.4-fold increase in CD40 expression (Figures 1a and 1b). This might reflect a TLR9-independent mechanism or the ability of the control ODN to stimulate TLR9 in BL cells.

To clarify this point, we used Akata cells overexpressing a dominant negative mutant of the adaptor protein MyD88 (DN-MyD88) [16]. Treatment with ODN CpG-2006 or ODN GpC-2006 induced increased CD40 expression, corroborating our first observation (Figure

1c). By contrast, neither ODN CpG-2006 nor ODN GpC-2006 significantly induced an increase in CD40 expression in DN-MyD88 Akata cells (Figure 1c). Finally, we validated the ODN CpG-2006 treatment-induced up-regulation of *STAT3* (Suppl. Table 1) by quantitative real-time polymerase chain reaction (qRT-PCR) and confirmed activation of STAT3 by Western blot (data not shown).

Collectively, we validated our microarray data showing that triggering with ODN CpG-2006 activates Akata cells to induce CD40 and STAT3 expression. Moreover, our results indicated that both ODNs used here act in a MyD88-dependent manner and that the ODN lacking CpG motifs activates Akata cells to a similar extent as the ODN containing CpG motifs.

TLR9 triggering by CpG ODN does not impact on the cell cycle of Akata cells. TLR9 triggering leads to cell cycle entry and proliferation of B chronic lymphatic leukemia cells[11, 12, 22]. We analyzed the cell cycle of mock-treated or ODN CpG-2006-treated Akata cells after initiation of treatment by propidium iodide staining and flow cytometry. Most cells were in the S phase during the time frame of 48 hours (Suppl. Figure 1). Treatment with ODN CpG-2006 did not alter the cell cycle compared to mock treatment. Similar results were obtained with Akata31 cells (data not shown), an EBV-negative subclone of Akata cells [23]. Thus, although TLR9 triggering by ODN CpG-2006 resulted in BL cell activation based on increased expression of certain genes, it did not seem to influence the cell cycle.

ODN-induced cell death of BL Akata cells is MyD88-dependent. Treatment with ODN GpC-2006 unexpectedly induced CD40 up-regulation in Akata cells in a MyD88-dependent manner similar to treatment with ODN CpG-2006. We determined whether treatment with distinct TLR9 ligands would similarly result in a MyD88-dependent effect on cell survival. Also, we explored whether a class A ODN, that displays low specificity for B-cells, exerts

similar effects on BL cells as class B ODNs that possess a high specificity for B-cells. Therefore, we treated Akata cells or DN-MyD88 Akata cells with ODN CpG-2216 (type A), ODN CpG-2006 (type B) and ODN GpC-2006 (type B control), respectively, and analyzed the viability of the cells by Trypan Blue exclusion assay and PE-Annexin V/7-AAD staining. The Trypan Blue exclusion assay showed that treatment of Akata cells with ODN CpG-2006 or ODN GpC-2006 reduced the survival of cells within 48 hours to below 50% of not treated cells and treatment with ODN CpG-2216 to almost 50% of not treated cells after 72 hours (Figure 2a). By contrast, the survival of DN-MyD88 Akata cells was not, if at all, reduced by treatment with any of the three ODNs for 72 hours (Figure 2a). These findings were corroborated by the Annexin V/7-AAD assay revealing that the viability of Akata cells was drastically affected by treatment with class B ODNs and to a lesser extent with class A ODN, contrasting DN-MyD88 Akata cells that were not affected by treatment with ODNs for 72 hours (Figure 2b and quantification in Figure 2c). Thus, treatment of Akata cells with ODNs CpG-2006 and GpC-2006 triggers TLR9 signaling via MyD88 and results in cell death.

ODN-induced cell death of BL Akata cells is caspase-dependent. To explore the mechanism of ODN-induced cell death of Akata cells we set out to investigate whether caspases were involved. Thus, the activity of caspase-3 and -7 in ODN-treated Akata cells was detected using a luminogenic substrate which gives rise to a luminescent signal proportional to caspase-3/7 activity. Triggering with TLR9 ligands for 48 hours increased the luminescent signal about 2-fold as compared to no triggering. This increase was abolished by treatment with the caspase inhibitor z-VAD-FMK (Figure 3a), indicating that TLR9 triggering resulted in increased caspase activity in Akata cells and that this activity was not increased if cells were concomitantly treated with a caspase inhibitor. These findings were corroborated using PI staining, which indicate that Akata cell death induced by treatment with ODNs can be abrogated by the caspase inhibitor z-VAD-FMK (Figure 3b); and by Western blotting

showing that PARP cleavage is increased by ODN treatment and can be reduced by the inhibitor (Figure 3c). Thus, ODN-induced cell death of Akata cells was found to be caspase-3/7 dependent and therefore due to apoptosis.

Survival of distinct BL cells following treatment with CpG ODNs differs considerably, independently of the presence of EBV. ODN CpG-2006 suppresses EBV lytic gene expression in Akata cells and primary B-cells promoting latent EBV that may provide cell survival signals[15, 16]. Since we observed Akata cell death upon TLR9 triggering, we asked whether the absence of EBV would result in even more pronounced cell death following TLR9 triggering. Hence, we tested whether TLR9 agonists affect the survival of EBV-positive Akata cells and of EBV-negative Akata31 cells in a similar fashion. Treatment with ODN CpG-2006 or ODN GpC-2006 strongly decreased the percentages of surviving Akata cells or Akata31 cells between 24 hours and 72 hours compared to untreated cells. Nevertheless, the effect on Akata31 cells was less marked (Figure 4a). The percentages of apoptotic Akata31 cells assessed by Annexin V/7-AAD staining after 72 hours of treatment, however, were comparable to those of Akata cells (Figure 4b). Treatment with ODN CpG-2216, ODN CpG-2006, or ODN GpC-2006 resulted in similar respective decreases in viability of Akata cells vs. Akata31 cells due to apoptosis. Treatment with class A ODN CpG-2216 exhibited almost no negative impact on cell survival compared to treatment with the class B ODNs CpG-2006 and GpC-2006 (Figure 4b). These results showed that EBV-negative Akata31 cells exhibited a higher level of spontaneous cell death by apoptosis compared to EBV-positive Akata cells and corroborated the lower effect of TLR9 triggering by class A ODN compared to triggering by class B ODNs. Moreover, the results suggested that the presence of EBV does not prevent EBV-positive Akata cells from TLR9 triggering-induced cell death compared to EBV-negative Akata31 cells.

Next, we investigated whether our observations in EBV-positive versus EBV-negative BL cells hold true in BL cells other than Akata. To this end, we used EBV-positive BL cells Mutu-I and EBV-negative BL cells BJAB and Ramos. Treatment with ODN CpG-2006 or with ODN GpC-2006 did not affect Mutu-I cell or BJAB cell survival (Figure 4c) but it decreased Ramos cell survival between 48 hours and 72 hours (Figure 4c). Thus, the survival of BL cells following TLR9 triggering considerably differs between distinct BL cells and does not seem to depend on the presence or absence of EBV.

Exogenous hIL-10 does not prevent ODN CpG-2006-induced cell death. hIL-10 plays an important role in B-cell lymphoma biology as it seems to act as autocrine growth factor [22, 24-26]. We previously reported [16] and corroborate here, a strong hIL-10 induction in Akata cells following TLR9 triggering with ODN CpG-2006 (Suppl. Table 1). Nevertheless, the very same treatment resulted in drastic Akata cell death (Figure 2), suggesting that the latter could not be prevented by the induction of hIL-10 expression. Otherwise, a recent report suggested that treatment with ODN CpG-2006 induced hIL-10 in chronic lymphocytic leukemia B-cells mediating their cell death [10]. Therefore, we treated Akata cells and DN-MyD88 Akata cells with ODN CpG-2006, recombinant hIL-10 (rhIL-10), or a combination thereof. The concentration of rhIL-10 was based on the peak hIL-10 protein concentration induced by ODN CpG-2006 treatment in Akata cells [16]. Additionally, ODN CpG-2006 treatment was reiterated at 48h in a fraction of BL cells.

As expected, treatment with ODN CpG-2006 for 72 hours reduced the viability of Akata cells to < 20% but did not reduce viability of DN-MyD88 Akata cells compared to no treatment (Figure 5a-c). Reiterated treatment with ODN CpG-2006 after 48 hours did not further reduce viability of Akata cells compared to one-time treatment and did not reduce viability of DN-MyD88 Akata cells (Figure 5a-c). Treatment with rhIL-10 for 72 hours also did not reduce viability of Akata cells or DN-MyD88 Akata cells compared to no treatment.

Treatment with rhIL-10 did not counteract ODN CpG-2006 treatment-induced reduction of BL cell viability or further decreased their viability. DN-MyD88 Akata cells were not affected by any of the treatments (Figure 5a-c). Thus, maximal negative impact of ODN CpG-2006 on cell survival in Akata cells occurred after one treatment. Moreover, rhIL-10 did not change the number of viable Akata cells, or rescue Akata cells from ODN CpG-2006 treatment-induced cell death. This suggested that hIL-10 cannot counteract TLR9 triggering-induced cell activation resulting in death of Akata cells and is not responsible for their cell death.

BL cells express distinct *hIL-10* mRNA and hIL-10 levels following TLR9 triggering. The distinct survival of BL cells following TLR9 triggering with ODN CpG could be due to distinct magnitudes of ensuing cellular responses. The induction of hIL-10 following ODN CpG treatment can be regarded as a surrogate marker for the magnitude of cell activation mediated by TLR9 triggering. Thus, we assessed *hIL-10* mRNA expression in BL cells treated with ODN CpG-2006 or ODN GpC-2006. All 5 BL cell lines tested expressed hIL-10 mRNA following treatment with ODN CpG-2006, the values peaking preferentially at 6h post treatment (Figure 6a). Peak *hIL-10* mRNA expression levels varied from 1.3 transcripts over *HMBS* in Ramos BL cells to 48 transcripts in Mutu-I BL cells. Treatment with ODN GpC-2006 also resulted in induction of *hIL-10* mRNA expression but in lower levels, except in Akata cells. Similar variation of results was observed when analyzing hIL-10 protein levels (Figure 6b). Thus, TLR9 triggering of distinct BL cells resulted in a broad range of *hIL-10* mRNA and hIL-10 protein expression levels, and the magnitude of the level seemed independent of EBV status.

Next, we determined *TLR9* mRNA expression levels in BL cells by qRT-PCR. Although distinct BL cells showed up to 6-fold different relative *TLR9* mRNA expression levels (Figure 6c), ODN CpG-2006 treatment-induced *hIL-10* mRNA peak expression levels did not correlate with the relative *TLR9* mRNA expression levels (Figure 6d). Collectively,

TLR9 triggering in distinct BL cells resulted in distinct *hIL-10* mRNA expression, not linked to presence or absence of EBV, and not correlated to *TLR9* mRNA expression levels.

***TLR9* polymorphisms of BL cells might correlate with distinct responses to TLR9 triggering.** *TLR9* polymorphisms in patients are linked to different outcomes of inflammatory diseases and the development of cancer [27, 28]. We hypothesized that *TLR9* polymorphisms could be a possible explanation for our observations. Thus, we isolated the genomic DNA from BL cell lines and analyzed it for the presence of *TLR9* polymorphisms. We found specific SNPs for each cell line, allowing segregation of the BL cell lines into three groups (Table 1): -1237 TT/1635 GA (Akata and Akata31), -1237 TT/1635 GG (Ramos) and -1237 TC/1635 GG (Mutu-I and BJAB). This sub-grouping correlated with the degree of BL cell survival in response to ODN treatment (Figure 4c), suggesting that the distinct BL cell responses to TLR 9 triggering may depend on the SNPs present.

Next, we analyzed *TLR9* SNPs in genomic DNA from primary BLs or blood from healthy individuals. Indeed, we found the *TLR9* SNPs detected in our BL cell lines in primary BL samples and blood of healthy individuals (Table 4). Importantly, the *TLR9* SNP frequencies were statistically not significantly different between BL cell lines, primary BLs and blood from healthy individuals, respectively. This suggested that if the direct response to TLR9 agonists depends on the *TLR9* SNP, this could be an important factor regarding the treatment of BL patients by TLR9 triggering.

Discussion

We analyzed the effects of TLR9 agonists on BL cell lines as an *in vitro* model for B-cell tumor. We found that treatment with TLR9 ligands induces distinct cytokine expression and cell death responses in distinct BL cells. Cell-death was (i) dependent on TLR9-MyD88 signaling; (ii) occurred concomitantly with activation and could be suppressed by pan caspase inhibitors; (iii) was not dependent on the presence or absence of EBV in the tumor cells; and (iv) was associated with SNPs in the *TLR9* gene. Our results suggest that individualized *in vitro* pretesting of BL responses to CpG ODNs could help to predict the outcome of therapeutic TLR9 triggering and tailor adjuvant molecular treatment of BL.

Our observation that BL cells of different origin show distinct cell survival following TLR9 triggering is novel. We previously demonstrated that TLR9 triggering counteracts lytic EBV reactivation in BL cells and promotes latent EBV that is associated with B-cell lymphoproliferation [16]. Here, we observed CpG type B ODN-induced cell death by apoptosis in EBV-positive Akata and EBV-negative Akata31 cells, but not or to a much lower extent in the other EBV-positive or EBV-negative BL cell lines tested. Thus, the EBV status of the tumor cells does not rule the responses of BL cells to TLR9 triggering.

TLR9-triggered BL cells consistently upregulated hIL-10 expression. hIL-10 influences the development and growth of B-cells [29] and acts as an autocrine growth factor for different B-cell lymphomas [25, 26, 30]. Importantly, here, rhIL-10 did neither prevent nor enhance cell death induced by TLR9 triggering. hIL-10, as an anti-inflammatory cytokine, inhibits the Th1 immune response [31] and this effect would be detrimental in cancer therapy that is based on intact or enhanced immune responses [9], since an unintended proliferation of the malignant cells could be provoked. Thus, the function of CpG type B ODN-induced hIL-10 expression in BL cells seems to considerably differ from that in chronic lymphocytic leukemia B-cells that were reported to undergo hIL-10-mediated apoptosis [10].

Surprisingly, ODN GpC-2006 induced cell death to the same extent as ODN CpG-2006. ODN GpC-2006 binds to TLR9, but in contrast to CpG-2006 it does not lead to TLR9 signaling in HEK293 cells or to changes in the secondary structure of the ectodomain [32]. Therefore, we examined whether downstream signaling of TLR9 via MyD88 is involved in the induction of cell death. Indeed, experiments using Akata cells overexpressing a MyD88 dominant negative mutant indicated that both ODNs induce cell death of BL cells in a MyD88-dependent manner. Others demonstrated that ODNs induce cell death independently of the CpG motif, of TLR9 [33] or even of the whole ODN sequence [34]. Another pathway that is triggered by CpG ODNs, but is TLR9-independent, was described in monocytes and involves the activation of the Src family kinases Lyn and Hck [35]. In our experiments, BL cell death induced by TLR9 triggering correlated with increased caspase activity and was caspase-dependent, thus most likely due to apoptosis.

The BL cell lines tested differed in their TLR9 mRNA expression level and TLR9 SNPs. mRNA expression did not correlate with cell death responses to TLR9 triggering, but the distinct TLR9 SNPs did. The role of TLR9 SNPs in BL is unknown. To start to investigate this we analyzed the frequencies of given SNPs in BL patients and healthy individuals and found them to be similar. A recent report suggests that the rs5743836 polymorphism, which was also detected here, confers an increased risk for non-Hodgkin lymphoma in people from Portugal and Italy but not from the US 20. The association of the C allele rs5743836 with lack of cell death upon TLR9 triggering observed here in the Mutu-I and BJAB BL cell lines is striking. It does not allow establishing a causal link, but justifies the hypothesis that the distinct cell death responses upon CpG ODN treatment in BL cells may be linked to TLR9 SNPs. Notably, the C allele of rs5743836 exhibits greater NF-κB binding affinity because of an additional NF-κB transcriptional binding site that may lead to increased production of proinflammatory cytokines 36. Hence, the presence of the C allele seems to result in enhanced

NF-κB activation following TLR9 triggering. This may lead to protection of ODN CpG treatment-induced apoptosis that can be observed in BL cells without the C allele.

In conclusion, therapeutic TLR9 triggering appears to be a double-edge sword that may induce apoptosis, or enhance lymphoproliferation. Our findings suggest that the effect of TLR9 agonists on BL cells should be tested *in vitro* prior to installment of therapy and that *TLR9* SNPs in BL patients should be evaluated as potential biological markers for the response to treatment targeting innate immunity.

Material and Methods

Cell lines. The EBV-positive BL cell lines Akata and Mutu-I, and the EBV-negative BL cell lines Akata 31, BJAB and Ramos were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, streptomycin (100 mg/ml), penicillin (100 U/ml) and L-glutamine (2mM). EBV-positive BL Akata cells expressing a dominant negative MyD88 (DN-MyD88 Akata) [16] were grown in the same medium supplemented with 0.4 mg/ml G418 (Promega, Mannheim, Germany).

Primary BL tissue samples and peripheral blood from healthy individuals. Burkitt's lymphomas were retrieved from the database of the Institute of Surgical Pathology, University Hospital of Zurich (PathoPro Software, Institute of Medical Software, Saarbrücken, Germany). The BL diagnosis was performed according to the WHO classification of tumors of hematopoietic and lymphoid tissues [37]. DNA was extracted from paraffin-embedded whole tissue sections, according to standard procedures. This study was in accordance with Swiss laws and approved by the official authorities of the ethical committee of the Canton of Zurich (StV2-2007). Peripheral blood was collected from 402 healthy blood donors (aged 19-70 years) who were randomly selected according to the criteria of the Swiss Red Cross [38].

Single-nucleotide-polymorphism analysis. Whole genomic DNA was extracted with the DNeasy® Blood & Tissue Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. Genotyping of the *TLR9* polymorphisms –1237 T/C (rs5743836) and 2848 G/A (rs352140) was done by tetra-primer assays as previously described [39].

Treatment with TLR9 agonists. Cells were split to a density of $0.5x10^6$ cells/ml. The TLR9 ligands ODN CpG-2216 (type A), ODN CpG-2006 (type B) and ODN GpC-2006 (type B control) were applied at concentrations of 0.5 µM. Oligodeoxynucleotide (LabForce-InvivoGen, Nunningen, Switzerland) sequences: ODN CpG-2216: 5'-ggGGGACGATCGTCgggggg-3', bases are phosphodiester (capital letters) or phosphorothioate (lower case); ODN CpG-2006: 5'-tcgtcgttttgtcgttttgtcgtt-3', full phosphorothioate backbone; ODN GpC-2006: 5'- tgctgcttttgtgcttttgtgctt -3', full phosphorothioate backbone.

Microarray analysis. Akata cells were mock treated or treated with CpG-2006, and harvested 6 hours later. Total RNA was isolated with RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Microarray analysis was performed at the Functional Genomic Centre Zurich (University of Zurich, Zurich, Switzerland). Human Exon 1.0 ST Array chips (Affymetrix, Santa Clara, CA, USA) and Genespring GX software (Agilent Technologies, Basel, Switzerland) were used for the analysis. An at least 2-fold change in gene expression (up-regulation or down-regulation) in the treated versus the mock-treated sample was regarded as significant.

qRT-PCR (Taqman). Total RNA was extracted from 10^6 cells with the RNeasy Kit (Qiagen) and DNA was removed with DNA-free (Ambion). 1 µg of the purified RNA was used to generate cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rotkreuz, Switzerland). Quantitative real-time PCR was performed with specific Taqman primers and probes on ABI 7200 (Applied Biosystems). Data were analyzed with the

software SDS2.2 (Applied Biosystems). Delta cycle threshold (Ct) values for the respective genes were normalized to *HMBS*.

Staining for surface antigens. 10^5 cells were stained with FITC mouse anti-human IgM (Clone G20-127), PE-Cy^{TM}5 mouse anti-human IgG (Clone G18-145), PE mouse anti-human IgD (Clone IA6-2) or the respective isotype controls, FITC IgG1κ isotype control (Clone MOPC-21), PE-Cy^{TM}5 mouse IgG1κ isotype control (Clone MOPC-21), PE mouse IgG2aκ isotype control (Clone G155-178) or PE-Cy5 anti-human CD40 (Cat. 555590) (all from BD Biosciences, Allschwil, Switzerland) for 30 min at 4 °C in the dark. Cells were counted with a flow cytometer (FACSCanto II, BD Biosciences).

Annexin V/7-AAD staining. To assess apoptotic, necrotic or dead and viable cells, 10^5 cells were harvested, washed with PBS and resuspended in 100 µl Annexin V binding buffer (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4) and incubated with 5 µl Annexin V and 5 µl 7-Amino-Actinomycin (7-AAD) (BD Biosciences) for 15 min at room temperature in the dark. After addition of 200 µl Annexin V binding buffer, cells were analyzed with the FACSCanto II flow cytometer (BD Biosciences).

Propidium Iodide staining. To discern between dead and viable cells, treated cells were stained with 50 µg/ml PI (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) for 15 min at room temperature and then analyzed with the FACSCanto II flow cytometer (BD Biosciences, Allschwil, Switzerland).

Protein isolation, quantification, and Western blot. Total protein lysates were obtained after lysing the cells in RIPA complete buffer [50 mM Tris–HCl pH7.5, 150 mM NaCl, 2 mM

EDTA, 1% NP40 complemented with 0.1% SDS, 1x EDTA-free protease inhibitor cocktail (Roche, Rotkreuz, Switzerland)]. Cell extracts were passed 10 times through a 25G syringe. Protein content was determines using the Pierce® BCA Protein Assay Kit (ThermoScientific, Erembodegem, Belgium), according to the manufacturer's instructions. To analyze protein expression by Western blot, protein (20µg/ per well) was loaded into a NuPAGE® 4-12% Bis-Tris Gel (life technologies, Zug, Switzerland) and subjected to SDS–PAGE electrophoresis, transferred electrophoretically to a nitrocellulose membrane (GE Healthcare, Glattbrugg, Switzerland), incubated with rabbit anti-PARP antibody or rabbit anti-β-Actin antibody, and subsequently with anti-rabbit IgG, HRP-linked antibody (all diluted 1:1000 and from, Cell Signaling Technology (Allschwil, Switzerland). The signal was detected with the ECL Western Blotting Detection Reagents (GE Healthcare) and imaged using the LAS-3000 (Fujifilm, Dielsdorf, Switzerland) and image reader LA-3000 (Fujifilm).

hIL-10 ELISA. hIL-10 protein concentrations were determined in supernatants from TLR9-stimulated cultures by standard capture ELISA (Ready-SET-Go, eBioscience, Vienna, Austria) according to the manufacturer's instructions Plates were read at 450 nm once the substrate had developed, and cytokine concentration was determined by extrapolation from the standard curve.

Caspase-3/7 activity. Activity of caspase-3/7 was assessed with the Caspase-Glo® 3/7 Assay according to the manufacturer's protocol (Promega, Wallisellen, Switzerland). Cells, dispensed at 0.5 x 10^6 cells/mL/well in a 24-well plate were treated with TLR9 ligands and incubated at 37°C for 48 hours. At the end of the incubation period, 100 µL of Caspase-Glo® 3/7 reagent was added to 100 µL cell suspension in a white-walled 96-well plate and

incubated at room temperature for 1 hour. Luminescence intensity was determined using a Synergy HT Multi-Detection Microplate Reader (BioTek, Luzern, Switzerland).

Treatment of BL cells with rhIL-10. One day before the experiment, the cells were split to a density of $0.5x10^6$cells/ml. The next day, 10^6 cells were incubated with 900 pg rhIL-10/ml (Immunotools, Friesoythe, Germany) for the indicated time points. This concentration corresponds to the hIL-10 protein concentration induced by ODN CpG-2006 in Akata cells [16].

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References

1. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 2004; **5:** 987-95.

2. Chiron D, Bekeredjian-Ding I, Pellat-Deceunynck C, Bataille R, Jego G. Toll-like receptors: lessons to learn from normal and malignant human B cells. *Blood* 2008; **112:** 2205-13.

3. Rifkin IR, Leadbetter EA, Busconi L, Viglianti G, Marshak-Rothstein A. Toll-like receptors, endogenous ligands, and systemic autoimmune disease. *Immunol Rev* 2005; **204:** 27-42.

4. Bourke E, Bosisio D, Golay J, Polentarutti N, Mantovani A. The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. *Blood* 2003; **102:** 956-63.

5. Dorner M, Brandt S, Tinguely M, Zucol F, Bourquin JP, Zauner L *et al.* Plasma cell toll-like receptor (TLR) expression differs from that of B cells, and plasma cell TLR triggering enhances immunoglobulin production. *Immunology* 2009; **128:** 573-9.

6. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T *et al.* Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* 2002; **168:** 4531-7.

7. Vollmer J, Krieg AM. Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. *Adv Drug Deliv Rev* 2009; **61:** 195-204.

8. Weiner GJ. CpG oligodeoxynucleotide-based therapy of lymphoid malignancies. *Adv Drug Deliv Rev* 2009; **61:** 263-7.

9. Krieg AM. Toll-like receptor 9 (TLR9) agonists in the treatment of cancer. *Oncogene* 2008; **27:** 161-7.

10. Liang X, Moseman EA, Farrar MA, Bachanova V, Weisdorf DJ, Blazar BR *et al.* Toll-like receptor 9 signaling by CpG-B oligodeoxynucleotides induces an apoptotic pathway in human chronic lymphocytic leukemia B cells. *Blood* 2010; **115:** 5041-52.

11. Jahrsdorfer B, Muhlenhoff L, Blackwell SE, Wagner M, Poeck H, Hartmann E *et al.* B-cell lymphomas differ in their responsiveness to CpG oligodeoxynucleotides. *Clin Cancer Res* 2005; **11:** 1490-9.

12. Decker T, Schneller F, Sparwasser T, Tretter T, Lipford GB, Wagner H *et al.* Immunostimulatory CpG-oligonucleotides cause proliferation, cytokine production, and an immunogenic phenotype in chronic lymphocytic leukemia B cells. *Blood* 2000; **95:** 999-1006.

13. Huang B, Zhao J, Li H, He KL, Chen Y, Chen SH *et al.* Toll-like receptors on tumor cells facilitate evasion of immune surveillance. *Cancer Res* 2005; **65:** 5009-14.

14. Wolska A, Lech-Maranda E, Robak T. Toll-like receptors and their role in carcinogenesis and anti-tumor treatment. *Cell Mol Biol Lett* 2009; **14:** 248-72.

15. Ladell K, Dorner M, Zauner L, Berger C, Zucol F, Bernasconi M *et al.* Immune activation suppresses initiation of lytic Epstein-Barr virus infection. *Cell Microbiol* 2007; **9:** 2055-69.

16. Zauner L, Melroe GT, Sigrist JA, Rechsteiner MP, Dorner M, Arnold M *et al.* TLR9 triggering in Burkitt's lymphoma cell lines suppresses the EBV BZLF1 transcription via histone modification. *Oncogene* 2010; **29:** 4588-4598.

17. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. *Nat Rev Cancer* 2004; **4:** 757-68.

18. Rickinson A, Kieff E. Epstein-Barr virus. In: Knipe DM (ed) *Fields Virology*, 4th edn. Lippincott Williams & Wilkins: Philadelphia, PA, 2001, pp 2575–2627.

19. Traggiai E, Becker S, Subbarao K, Kolesnikova L, Uematsu Y, Gismondo MR *et al.* An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nat Med* 2004; **10:** 871-5.

20. Carvalho A, Cunha C, Almeida AJ, Osorio NS, Saraiva M, Teixeira-Coelho M *et al.* The rs5743836 polymorphism in TLR9 confers a population-based increased risk of non-Hodgkin lymphoma. *Genes Immun* 2012; **13**:197-210.

21. Peng SL. Signaling in B cells via Toll-like receptors. *Curr Opin Immunol* 2005; **17:** 230-6.

22. Jahrsdorfer B, Hartmann G, Racila E, Jackson W, Muhlenhoff L, Meinhardt G *et al.* CpG DNA increases primary malignant B cell expression of costimulatory molecules and target antigens. *J Leukoc Biol* 2001; **69:** 81-8.

23. Jenkins PJ, Binne UK, Farrell PJ. Histone acetylation and reactivation of Epstein-Barr virus from latency. *J Virol* 2000; **74:** 710-20.

24. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001; **19:** 683-765.

25. Beatty PR, Krams SM, Martinez OM. Involvement of IL-10 in the autonomous growth of EBV-transformed B cell lines. *J Immunol* 1997; **158:** 4045-51.

26. Jones KD, Aoki Y, Chang Y, Moore PS, Yarchoan R, Tosato G. Involvement of interleukin-10 (IL-10) and viral IL-6 in the spontaneous growth of Kaposi's sarcoma herpesvirus-associated infected primary effusion lymphoma cells. *Blood* 1999; **94:** 2871-9.

27. Ng MT, Van't Hof R, Crockett JC, Hope ME, Berry S, Thomson J *et al.* Increase in NF-kappaB binding affinity of the variant C allele of the toll-like receptor 9 -1237T/C polymorphism is associated with Helicobacter pylori-induced gastric disease. *Infect Immun*; **78:** 1345-52.

28. Soriano-Sarabia N, Vallejo A, Ramirez-Lorca R, Rodriguez Mdel M, Salinas A, Pulido I *et al.* Influence of the Toll-like receptor 9 1635A/G polymorphism on the CD4 count, HIV viral load, and clinical progression. *JAIDS (1999)* 2008; **49:** 128-35.

29. Rousset F, Garcia E, Defrance T, Peronne C, Vezzio N, Hsu DH *et al.* Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc Natl Acad Sci U S A* 1992; **89:** 1890-3.

30. Masood R, Zhang Y, Bond MW, Scadden DT, Moudgil T, Law RE *et al.* Interleukin-10 is an autocrine growth factor for acquired immunodeficiency syndrome-related B-cell lymphoma. *Blood* 1995; **85:** 3423-30.

31. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol* 2010; **10:** 170-81.

32. Latz E, Verma A, Visintin A, Gong M, Sirois CM, Klein DC *et al.* Ligand-induced conformational changes allosterically activate Toll-like receptor 9. *Nat Immunol* 2007; **8:** 772-9.

33. Grauer OM, Molling JW, Bennink E, Toonen LW, Sutmuller RP, Nierkens S *et al.* TLR ligands in the local treatment of established intracerebral murine gliomas. *J Immunol* 2008; **181:** 6720-9.

34. Castro JE, Prada CE, Aguillon RA, Kitada S, Fukuda T, Motta M *et al.* Thymidine-phosphorothioate oligonucleotides induce activation and apoptosis of CLL cells independently of CpG motifs or BCL-2 gene interference. *Leukemia* 2006; **20:** 680-8.

35. Sanjuan MA, Rao N, Lai KT, Gu Y, Sun S, Fuchs A *et al.* CpG-induced tyrosine phosphorylation occurs via a TLR9-independent mechanism and is required for cytokine secretion. *J Cell Biol* 2006; **172:** 1057-68.

36. Ng MT, Van't Hof R, Crockett JC, Hope ME, Berry S, Thomson J *et al.* Increase in NF-kappaB binding affinity of the variant C allele of the toll-like receptor 9 -1237T/C polymorphism is associated with Helicobacter pylori-induced gastric disease. *Infect Immun* 2010; **78:** 1345-52.

37. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H *et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, IARC: Lyon, 2008.

38. Muntwyler J, Marti-Jaun J, Luscher TF, Hanseler E, Hersberger M. The Asp298 but not the C-786 genotype of the endothelial nitric oxide synthase is reduced with age in healthy Swiss men. *Clinical chemistry and laboratory medicine : CCLM / FESCC* 2005; **43:** 971-3.

39. Krayenbuehl PA, Hersberger M, Truninger K, Mullhaupt B, Maly FE, Bargetzi M *et al.* Toll-like receptor 4 gene polymorphism modulates phenotypic expression in patients with hereditary hemochromatosis. *Eur J Gastroenterol Hepato.* 2010; **22:** 835-41.

Table 1. TLR9 single nucleotide polymorphisms in the five investigated BL cell lines

		Single nucleotide polymorphism	
		rs5743836	**rs352140**
BL cell line	**Epstein-Barr virus**	**-1237 T/C**	**2848 G/A (P545P)**
Akata	Positive	T/T	G/A
Akata 31	Negative	T/T	G/A
Ramos	Negative	T/T	G/G
Mutu-I	Positive	T/C	G/G
BJAB	Negative	T/C	G/G

Table 2. Frequencies of *TLR9* single nucleotide polymorphisms in five BL cell lines, 15 primary BL tumors, and blood samples from 402 healthy individuals

Single nucleotide polymorphism				
rs5743836 -1237 T/C	rs352140 2848 G/A (P545P)	BL cell lines N	BL tumors N (%)	Healthy individuals N (%)
T/T	G/G	1	4 (27)	178 (44)
T/T	G/A	2	5 (33)	165 (41)
T/T	A/A	-	2 (13)	-
T/C	G/G	-	-	1 (0)
T/C	G/A	2	1 (7)	55 (14)
T/C	A/A	-	3 (20)	5 (1)

The frequencies of the polymorphisms were statistically not significantly different

Titles and legends to figures

Figure 1 TLR9 agonists induce CD40 expression in Akata cells in a MyD88-dependent manner. Akata cells or DN-MyD88 Akata cells were left untreated or treated with 0.5 μM ODN CpG-2006 or ODN GpC-2006 for 48 hours. After 6 hours, 24 hours and 48 hours, 10^5 cells were harvested, washed with PBS and stained with PE-Cy5-conjugated mouse anti-human CD40 antibody or a PE-Cy5 mouse IgG1κ isotype control and analyzed with a flow cytometer. (a) FACS plots of Akata cells. Isotype control: shaded grey line; anti-CD40 PE-Cy5: black line. Numbers indicate the mean fluorescence intensity (MFI) (b) Quantification of (a). (c) Comparison of CD40 expression in Akata cells and DN-MyD88 Akata cells.

Figure 2 The cell death induced by TLR9 agonists is MyD88 dependent. Akata cells and DN-MyD88 Akata cells were incubated with medium only, ODN CpG-2216, ODN CpG-2006, or ODN GpC-2006. (a) The cell survival was assessed by Trypan Blue exclusion assay 0 hours, 24 hours, 48 hours, and 72 hours after treatment. (b) Apoptosis assay after 72 hours of treatment. 10^5 cells were stained with PE-Annexin V/ 7-AAD and analyzed by flow cytometry. The double-negative population represents viable cells while PE-Annexin V positive or double-positive populations represent non-viable cells. (c) Quantification of the Annexin V/7-AAD assay in (b). Shown are the results of a representative experiment of two experiments (showing mean values).

Figure 3 TLR9 ligands induce cell death of Akata cells via caspase activation. (a) Caspase activity in 0.5 x 10^6 Akata cells measured 48 hours after treatment with the TLR9 ligands ODN CpG-2006, ODN GpC-2006 and ODN CpG-2216 without or with z-VAD-FMK. Cells were incubated with the Caspase-Glo luminogenic substrate to detect activated caspases and analyzed with a luminometer. Results represent the relative light units generated by active caspases 3 and 7. Shown in the bar graph are the results of a representative experiment out of

three performed in duplicate. (b) Treatment of Akata cells with caspase inhibitor z-VAD-FMK reduces TLR9 ligand-induced cell death. 10^6 Akata cells were treated with the TLR9 ligands ODN CpG-2006, ODN GpC-2006 or ODN CpG-2216 with or without addition of z-VAD-FMK. After 48 hours cells were stained with PI, and analyzed by flow cytometry. Shown are the results of a representative experiment out of two experiments performed in duplicate. (c) Treatment of Akata cells with TLR9 ligand induces PARP cleavage. Lysates from 10^6 Akata cells 48 hours after incubation with the TLR9 ligands ODN CpG-2216, ODN CpG-2006 and ODN GpC-2006 with or without z-VAD-FMK were analyzed by immunoblotting for PARP cleavage. The relative density of the cleaved PARP was calculated using densitometry and normalized to β-actin.

Figure 4 TLR9 agonists induce cell death of Akata cells and Akata 31 BL cells, but not of Mutu-I BL cells or BJAB BL cells, while Ramos BL cells are intermediately affected. 10^6 cells were treated with the TLR9 ligands ODN CpG-2216, ODN CpG-2006 and ODN GpC-2006. (a) Survival of untreated, ODN CpG-2006 treated or ODN GpC-2006 treated Akata cells and Akata 31 BL cells was assessed by Trypan Blue exclusion assay and counting of viable and dead cells and compared to the untreated control. (b) Apoptosis of Akata and Akata 31 BL cells after 72 hours of treatment. 10^5 cells were stained with PE-Annexin V/7-AAD and analyzed by flow cytometry (left panel); percentages of PE-Annexin V/7-AAD negative cells (right panel). (c) The survival of untreated, ODN CpG-2006 or ODN GpC-2006 treated Mutu-I, BJAB, and Ramos BL cells was evaluated by Trypan Blue exclusion. Shown are the results of a representative experiment out of at least two experiments performed in duplicate (showing mean values) or in triplicate (showing mean values $\pm$ s.e.m., indicated by error bars).

Figure 5 hIL-10 does not influence ODN CpG-2006-induced cell death. Akata cells and DN-MyD88 Akata cells were incubated with medium only, 0.5 µM ODN CpG-2006, 900 pg/ml rhIL-10, or combination of ODN CpG-2006 and rhIL-10. To one ODN CpG-2006-stimulated sample, 0.5 mM ODN CpG-2006 was added again after 48 hours (rep. CpG-2006). (A) The cell viability was monitored by the Trypan Blue exclusion test 0 hours, 48 hours, and 72 hours after treatment. (b) After 72 hours cells were harvested and 10^5 cells were stained with PE-Annexin V/7-AAD and analyzed by flow cytometry. (c) Quantification of (b).

Figure 6 Treatment of BL cell lines with TLR9 agonists induces distinct *hIL-10* mRNA and hIL-10 expression levels. (a) TLR9 triggering results in a wide range of *hIL-10* mRNA expression levels. BL cells were left untreated or treated with 0.5 µM ODN CpG-2006 or ODN GpC-2006 for the indicated time points. (b) hIL-10 protein expression levels in the culture supernatants of BL cell lines 6 hours after treatment or no treatment as in (a) measured by ELISA. (c) *TLR9* mRNA expression levels differ between BL cell lines by up to 6-fold. EBV positive (black bars) and EBV negative (grey bars) BL cells. (d) The *hIL10* mRNA peak expression responses to ODN CpG-2006 did not correlate with the corresponding relative *TLR*9 mRNA levels. Total RNA of 10^6 cells was isolated and transcribed into cDNA. *HMBS*, *hIL-10* or *TLR9* mRNA expression levels were determined by qRT-PCR. The Ct values for *hIL-10* and *TLR9* were normalized to the Ct values of the housekeeping gene *HMBS*. Maximal values of relative *hIL-10* mRNA expression were correlated with the respective relative *TLR9* mRNA expression using a two-tailed Pearson correlation. Shown are the results of a representative experiment out of at least two experiments performed in duplicate (showing mean values).

Figure 1

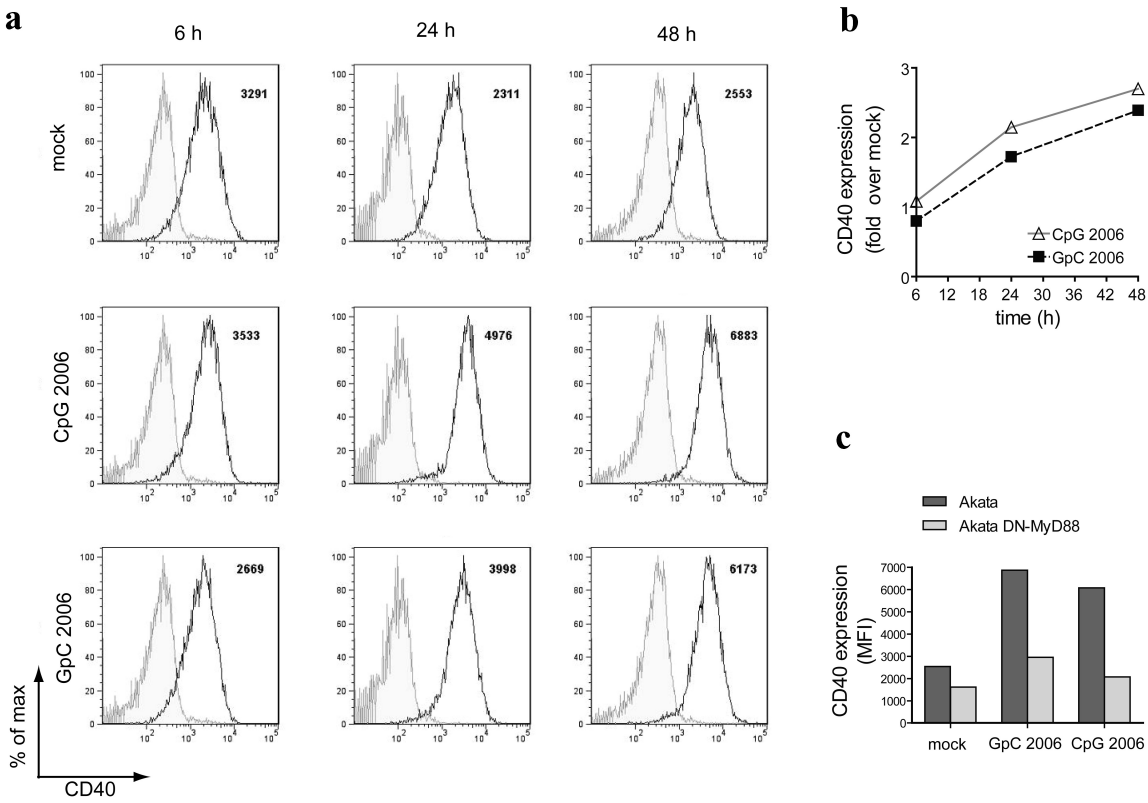

a
6 h
24 h
48 h
mock
CpG 2006
GpC 2006
3291
2311
2553
3533
4976
6883
2669
3998
6173
% of max
CD40
b
CD40 expression (fold over mock)
CpG 2006
GpC 2006
time (h)
c
Akata
Akata DN-MyD88
CD40 expression (MFI)
mock
GpC 2006
CpG 2006

Figure 2:

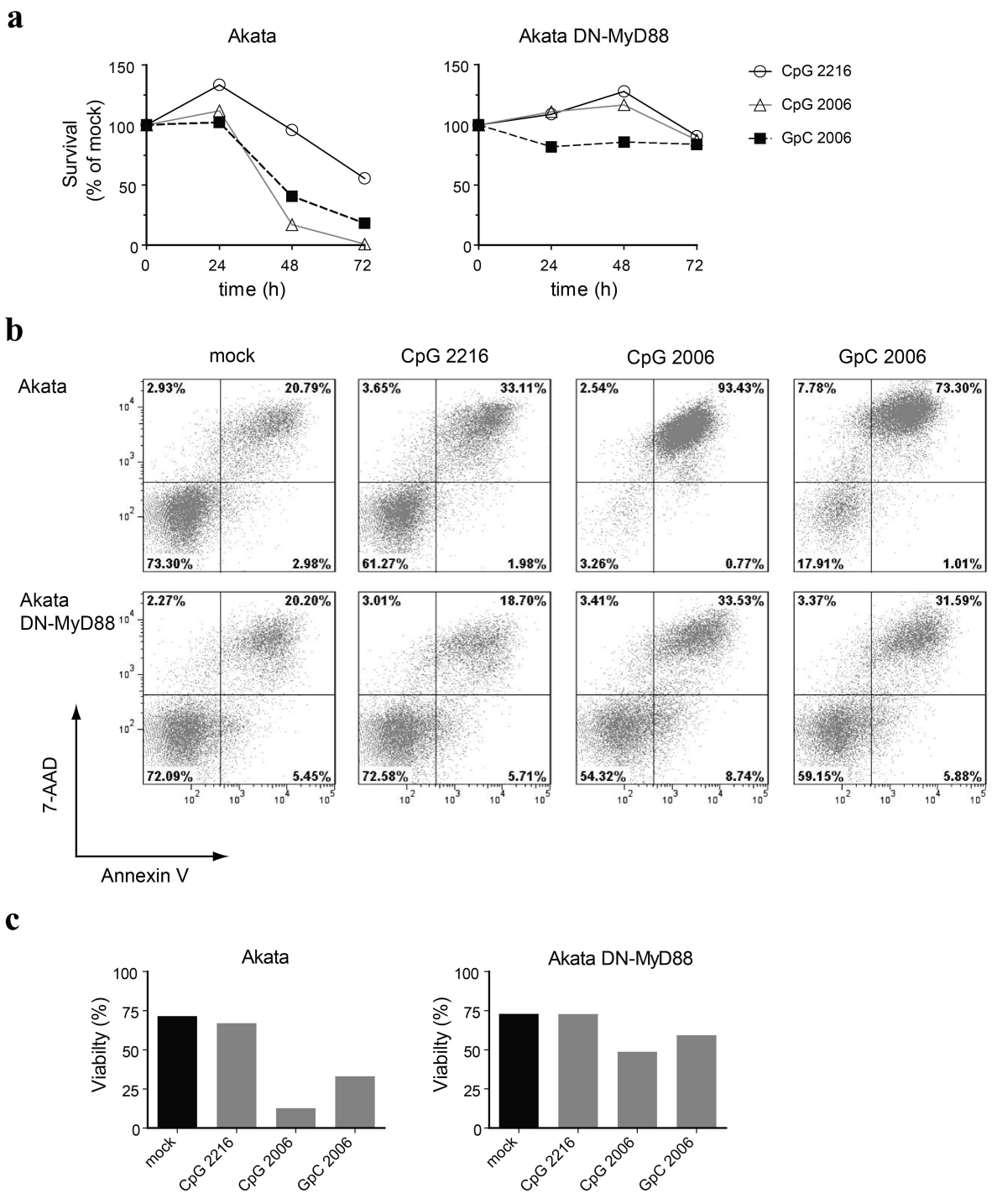

a
Akata
Akata DN-MyD88
Survival (% of mock)
time (h)
CpG 2216
CpG 2006
GpC 2006
b
mock
CpG 2216
CpG 2006
GpC 2006
Akata
Akata DN-MyD88
2.93%
20.79%
73.30%
2.98%
3.65%
33.11%
61.27%
1.98%
2.54%
93.43%
3.26%
0.77%
7.78%
73.30%
17.91%
1.01%
2.27%
20.20%
72.09%
5.45%
3.01%
18.70%
72.58%
5.71%
3.41%
33.53%
54.32%
8.74%
3.37%
31.59%
59.15%
5.88%
7-AAD
Annexin V
c
Akata
Akata DN-MyD88
Viability (%)
mock
CpG 2216
CpG 2006
GpC 2006

Figure 3

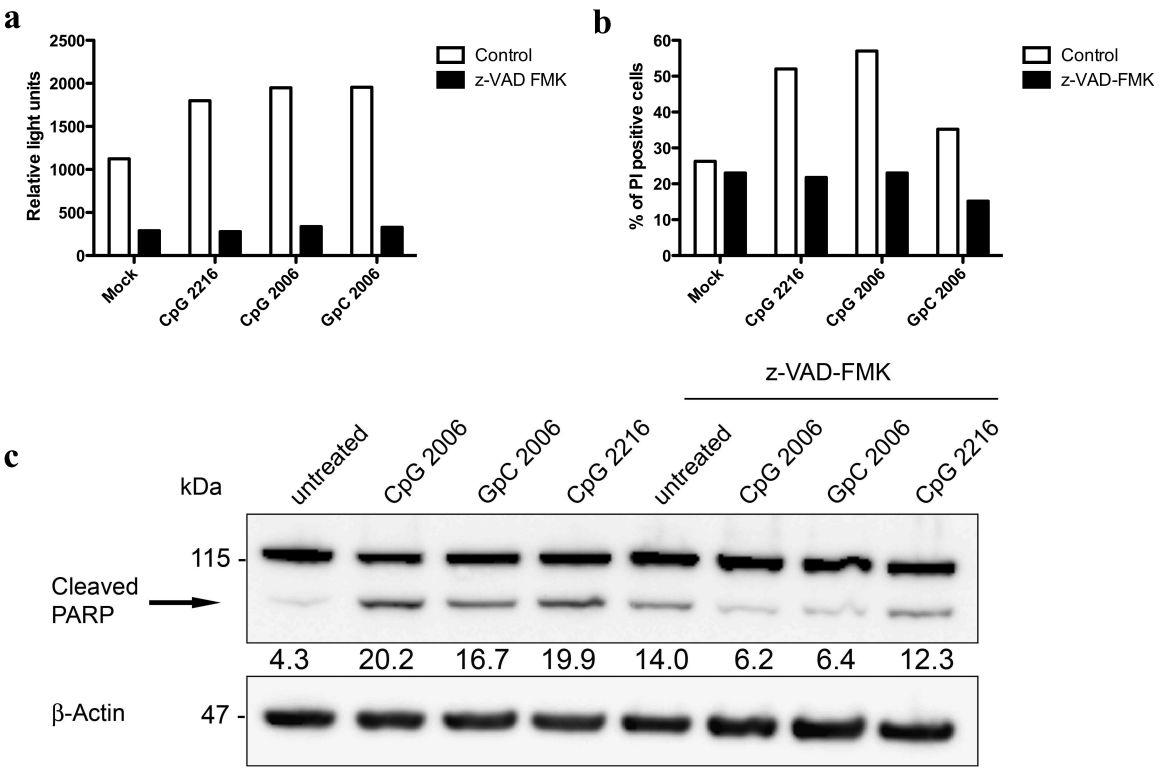

a
Relative light units
2500
2000
1500
1000
500
0
Control
z-VAD FMK
Mock
CpG 2216
CpG 2006
GpC 2006
b
% of PI positive cells
60
50
40
30
20
10
0
Control
z-VAD-FMK
Mock
CpG 2216
CpG 2006
GpC 2006
c
z-VAD-FMK
kDa
untreated
CpG 2006
GpC 2006
CpG 2216
untreated
CpG 2006
GpC 2006
CpG 2216
115
Cleaved
PARP
4.3
20.2
16.7
19.9
14.0
6.2
6.4
12.3
β-Actin
47

Figure 4

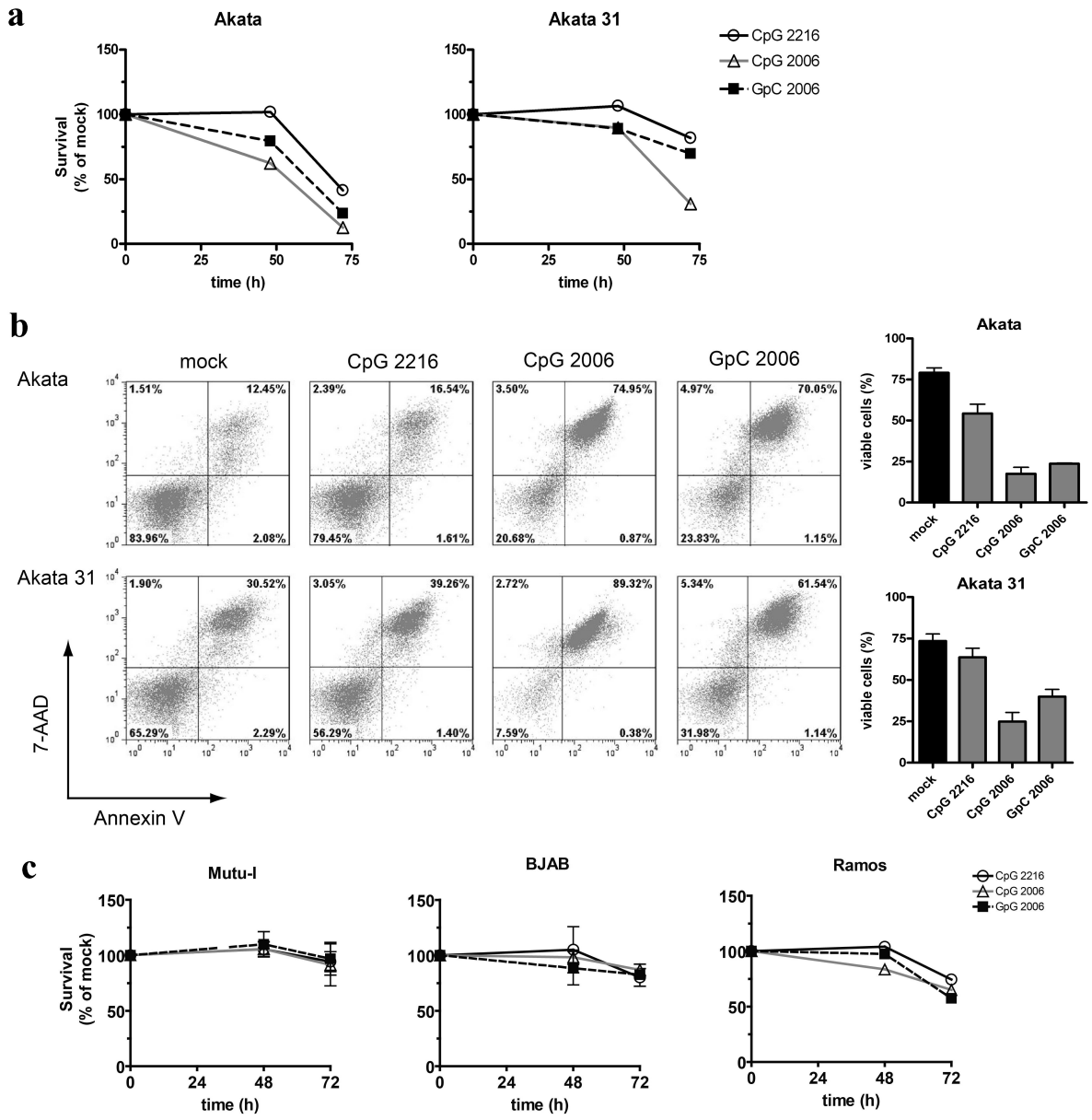

a
Akata
Akata 31
CpG 2216
CpG 2006
GpC 2006
Survival (% of mock)
time (h)
b
mock
CpG 2216
CpG 2006
GpC 2006
Akata
Akata 31
1.51%
12.45%
83.96%
2.08%
2.39%
16.54%
79.45%
1.61%
3.50%
74.95%
20.68%
0.87%
4.97%
70.05%
23.83%
1.15%
1.90%
30.52%
65.29%
2.29%
3.05%
39.26%
56.29%
1.40%
2.72%
89.32%
7.59%
0.38%
5.34%
61.54%
31.98%
1.14%
7-AAD
Annexin V
viable cells (%)
mock
CpG 2216
CpG 2006
GpC 2006
c
Mutu-I
BJAB
Ramos
Survival (% of mock)
time (h)

Figure 5

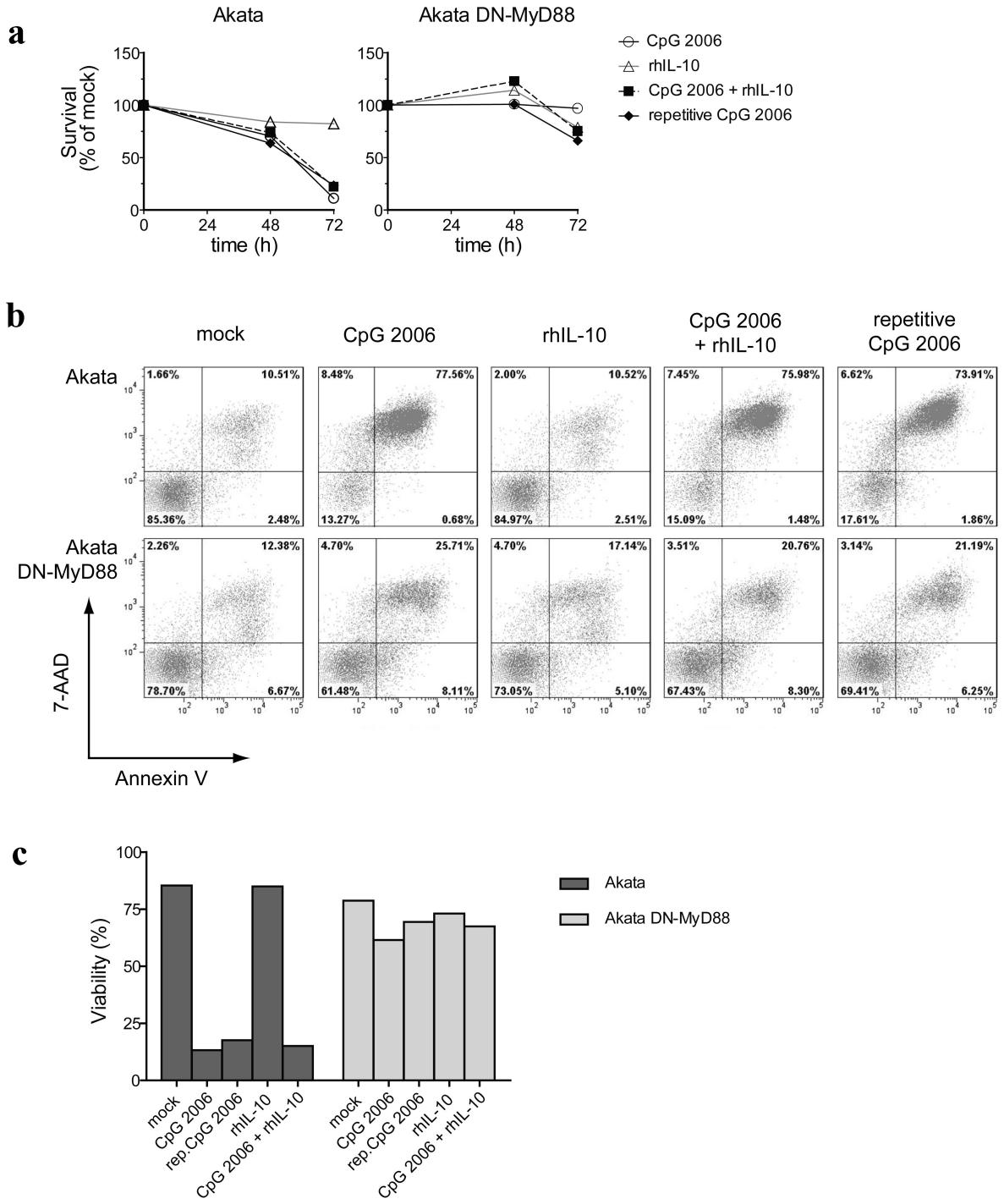
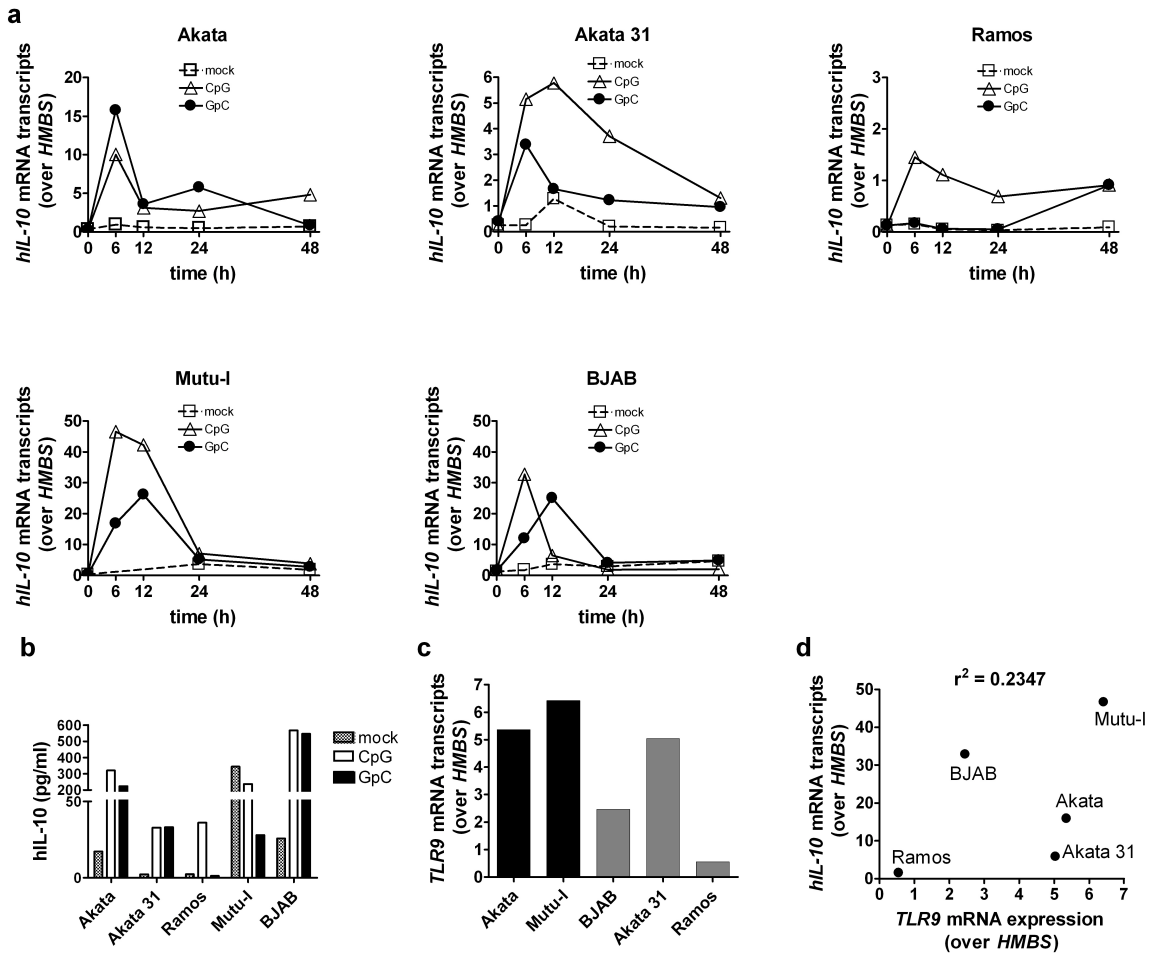
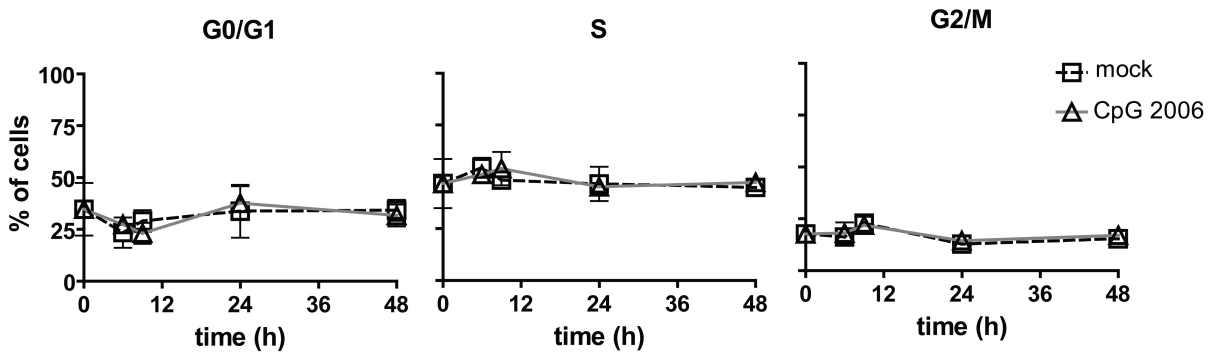

a
Akata
Akata DN-MyD88
Survival (% of mock)
time (h)
CpG 2006
rhIL-10
CpG 2006 + rhIL-10
repetitive CpG 2006
b
mock
CpG 2006
rhIL-10
CpG 2006 + rhIL-10
repetitive CpG 2006
Akata
Akata DN-MyD88
7-AAD
Annexin V
c
Viability (%)
Akata
Akata DN-MyD88
mock
CpG 2006
rep.CpG 2006
rhIL-10
CpG 2006 + rhIL-10

Figure 6



Cell cycle analysis

DNA of treated or untreated cells was measured by staining with propidium iodide (PI) and subsequent flow cytometric analysis. 10^6 cells were harvested, washed with PBS and fixed and permeabilized with cold 70% ethanol. The fixed and permeabilized cells were stored at 4 °C for 1h to one week. Before the staining, cells were washed with 1xPBS and resuspended in 200 µl PI-staining solution: 0.1% Triton X-100 (Sigma-Aldrich, Buchs, Switzerland), 0.2 mg/ml RNase A (Qiagen), 2 mg/ml PI (Sigma-Aldrich) in PBS. Cells were incubated at room temperature for 30 min and analyzed by flow cytometry (FACSCanto II, BD Biosciences).



Suppl Fig 1. TLR9 triggering with ODN CpG 2006 does not impact on the cell cycle of Akata BL cells. 10^6 cells were treated with or without 0.5 mM ODN CpG 2006 for the indicated time points, washed and fixed and permeabilized with cold 70 % Ethanol. The DNA content of the cells was measured by staining with propidium iodide and subsequent flow cytometric analysis. The frequencies of the cell cycles stages of the living cells were calculated using the Watson algorithm of the software FlowJo 7.6. Data represent the average of three independent experiments.

Suppl. Table 1. Selection of up-regulated genes in microarray analysis of ODN CpG 2006 treated versus untreated Akata BL cells.

GenBank gene symbol	Name	Fold change over untreated
Cytokines		
hIL-10	human interleukin 10	3.901
CXCL10	chemokine (C-X-C motif) ligand 10	3.543
B cell activation		
CD40	cluster of differentiation 40	3.011
CD69	cluster of differentiation 69	4.008
CD80	cluster of differentiation 80/ B7.1	2.833
CD83	cluster of differentiation 83	4.996
CD86	cluster of differentiation 86/ B7.2	2.340
Transcription factors		
CIITA	class II, major histocompatibility complex, transactivator	2.354
STAT3	signal transducer and activator of transcription 3	2.738
STAT5A	signal transducer and activator of transcription 5A	3.038
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	3.018
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	3.091
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	2.740
NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	2.124
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	8.414
Apoptosis		
FAS	Fas Receptor/ TNF receptor superfamily, member 6/Apo-1/ CD95	3.837
BCL2A1	Bcl-2-related protein A1	3.384

Fold change over untreated ≥ 2.0, $p \leq 0.05$.

Suppl. Table 2: Down-regulated genes in microarray analysis of ODN CpG 2006 treated versus untreated Akata BL cells.

GenBank gene symbol	Name	Fold change over untreated
B cell differentiation		
AICDA	activation-induced cytidine deaminase	2.413
Transcription factor		
BTBD3	BTB (POZ) domain containing 3	2.362
Cell cycle		
C13orf5	chromosome 13 open reading frame 15	2.214
CAB39L	calcium binding protein 39-like	2.714
Apoptosis		
RNF144B	ring finger 144B	2.116
Other		
SH2D1A	SH2 domain protein 1A, Duncan's disease (lymphoproliferative syndrome) positive regulation of natural killer cell mediated cytotoxicity	2.817
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4: response to unfolded protein	2.341
SLAMF6	SLAM family member 6: receptor activity	2.207
USP53	ubiquitin specific peptidase 53: ubiquitin-dependent protein catabolic process	2.518
Unknown function		
IGLV6-57	immunoglobulin lambda variable 6-57	2.471
LOC253012		2.320
SUSD3	sushi domain containing 3	2.006
CCDC144	coiled-coil domain containing 144 | TL132 protein	2.265

Fold change over untreated ≤ 2.0, $p \leq 0.05$.

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A doctoral thesis is so much more than just the sum of all the experiments performed. It would not have been possible to reach this point without the great support of many people. Therefore I would like to thank all the people who guided and supported me during my time as Ph.D. student, in particular:

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